AN ABSTRACT OF THE DISSERTATION OF

Angela Mastaloudis for the degree of Doctor of Philosophy in Exercise and Sport Science presented on April 13, 2004.
Title: Inhibition of Exercise-Induced Oxidative Stress, Inflammation and Muscle Damage by Prior Supplementation with the Antioxidant Vitamins E and C.

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Abstract approved:

Maret G. Traber

To determine if 6 weeks prior supplementation with vitamins E and C could alleviate exercise-induced DNA damage, prevent lipid peroxidation and inflammation, slow the rate of vitamin E utilization and/or attenuate muscle damage, 22 subjects (11 females: 11 males) were studied during a 50 km (32 mile) ultramarathon. Subjects were randomly assigned to treatment groups: placebos (PL) or antioxidants (AO, vitamin E (300 mg *RRR*-α-tocopheryl acetate) and 1000 mg vitamin C (500 mg twice per day)). Subjects consumed deuterium-labeled vitamin E (75 mg d_6-*RRR*-α-tocopheryl acetate) 48 h prior to race start. Multiple blood samples were obtained for measurements of DNA damage, unlabeled and labeled α-tocopherols and their metabolites, ascorbic acid, markers of lipid peroxidation (plasma F_2-isoprostanes (F_2-IsoPs)), inflammation and muscle damage. Maximal voluntary contraction (MVC) of the knee flexors/extensors was assessed. Plasma α-tocopherol and ascorbic acid increased in the AO but not the PL group following 6 weeks of supplementation. All subjects completed the race; average run time 7.1 ± 0.1 h. The run induced non-persistent DNA damage; % DNA damage increased at mid-race (p<0.02), but returned to baseline by 2 h post-race. One day post-

race, AO women had 62% less DNA damage than PL women (p<0.0008). Although F_2-IsoP levels were similar between groups at baseline, F_2-IsoPs increased during the run *only* in the PL group (from 28 ± 2 to 41 ± 3 pg/ml). Markers of inflammation increased in response to the run regardless of treatment. α-Tocopherol disappeared faster during exercise compared with rest, in women compared with men and in AO supplemented compared with PL subjects. Substantial MVC force deficits and increases in muscle damage markers post-race were similar between treatment groups. The ultramarathon run elicited oxidative stress, muscle damage and inflammation. Supplementation with vitamins E and C completely inhibited exercise-induced lipid peroxidation and appeared to benefit women with respect to DNA damage during recovery, but had no effect on exercise-induced inflammation, or muscle damage markers. Thus, vitamin E and C supplements are beneficial in preventing lipid peroxidation, but not the other adverse consequences of extreme exercise.

Inhibition of Exercise-Induced Oxidative Stress, Inflammation and Muscle
Damage by Prior Supplementation with the Antioxidant Vitamins E and C

by
Angela Mastaloudis

A DISSERTATION

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APPROVED: *Redacted for Privacy*

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Major Professor, representing Exercise and Sport Science

Redacted for Privacy

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Chair of the Department of Exercise and Sport Science

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Angela Mastaloudis, Author

ACKNOWLEDGEMENTS

CONTRIBUTION OF AUTHORS

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CHAPTER 1: INTRODUCTION

Despite the many known health benefits of exercise including cardiovascular fitness, blood glucose control, maintenance of lean body mass, and positive effects on the lipid profile, there is a wide body of evidence suggesting that exercise results in oxidative stress. Vigorous exercise causes oxidative stress resulting in lipid peroxidation (1-13) and DNA damage (14-19). Evidence of protein oxidation resulting from exercise-induced oxidative stress is less definitive (20-22). It is generally accepted that at rest the body continuously produces reactive oxygen species (ROS), yet in healthy individuals at rest, these ROS are produced at levels well within the capacity of the body's antioxidant defense system. In response to endurance exercise, oxygen (O_2) consumption increases 10 to 20-fold systemically and as much as 100 to 200-fold at the level of the skeletal muscle resulting in substantially increased mitochondrial electron flux (6). Leakage of electrons from the mitochondrial electron transport chain is considered a main source of reactive oxygen species (ROS) during exercise (23). Other potential sources of ROS during exercise include enhanced purine oxidation, damage to iron-containing proteins, disruption of Ca^{2+} homeostasis (24) and neutrophil activation (8). Exercise elicits oxidative stress as production of ROS outpaces antioxidant defenses (23). Furthermore, the acute phase inflammatory response elicited by exercise-induced muscle damage can be modulated by ROS (25).

Previously, we demonstrated that lipid peroxidation increases in response to endurance exercise (1). A nearly two-fold increase in F_2-isoprostanes (F_2-IsoPs), a sensitive marker of lipid peroxidation, was observed

immediately following a 50 km ultramarathon. In addition, the disappearance of deuterium labeled α-tocopherol was accelerated in response to the run offering further evidence for exercise-induced oxidative damage in excess of antioxidant defenses.

α-Tocopherol acts to protect polyunsaturated fatty acids (PUFA) in biological membranes against lipid peroxidation as it is the antioxidant primarily responsible for scavenging peroxyl radicals (26). Therefore, vitamin E supplementation might alleviate exercise-induced lipid peroxidation. Ascorbic acid is considered to be the most effective antioxidant in human plasma (27) as it is the only antioxidant that can prevent initiation of lipid peroxidation induced by aqueous peroxyl radicals (28). Ascorbate also spares other antioxidants including α-tocopherol and urate (27). The potent effects of ascorbic acid against ROS and its ability to spare α-tocopherol make it another logical source of protection against exercise-induced oxidative stress.

Hypothetically, supplementation with vitamins E and C could alleviate exercise-induced oxidative stress. Results from studies investigating the protective effects of supplementation with vitamins E and C alone or in combination against exercise-induced lipid peroxidation have been inconclusive. There have been reports of inhibition of lipid peroxidation (29-35), no effect (36-40) and even increased lipid peroxidation (32, 41) in response to supplementation. Possible reasons for the inconsistent findings include: differences in modes, duration, and intensity of exercise as well as variation in the methodologies used to assess lipid peroxidation. Other confounders include discrepancies in the types, amounts and bioavailability of antioxidant supplements provided, and in the duration of supplementation. A lack of consensus regarding the effectiveness of antioxidants to inhibit

exercise-induced lipid peroxidation and the popularity of antioxidant supplements in the physically active community make this an important area for research (42).

HYPOTHESES AND SPECIFIC AIMS

The generation of oxidative stress by exercise in humans and the potential for its amelioration by antioxidants is the basis for this research. We have demonstrated that endurance exercise causes an increase in oxidative stress (1). This level of exercise apparently produces oxidative damage in excess of antioxidant defenses. Furthermore, damage to skeletal muscle cell membranes by ROS can impair cell viability leading to necrosis and an acute phase inflammatory response (25, 43). Therefore, the hypothesis tested is that prior supplementation with the antioxidant vitamins E and C protects against the oxidative damage, inflammation and muscle damage generated during endurance exercise.

Specific Aim 1. Can endurance exercise-induced oxidative stress be prevented by prior antioxidant supplementation?

A primary purpose of the present study was to determine whether supplementation with vitamins E and C could prevent exercise-induced oxidative stress. Oxidative damage was assessed by changes in lipid peroxidation (F_2-IsoPs) and DNA damage (Comet assay) in response to endurance exercise.

Specific Aim 2. If supplementation with vitamins E and C attenuate exercise-induced oxidative stress, is vitamin E turnover reduced?

A second goal of this study was to evaluate whether decreasing oxidative stress by prior supplementation with the antioxidants (vitamins E and C) could moderate the rate of disappearance of deuterium labeled α-tocopherol (vitamin E turnover).

Specific Aim 3. Can prior supplementation with vitamins E and C modulate the inflammatory response to endurance exercise?

Due to a proposed relationship between oxidative stress and inflammation, another aim of the present study was to determine whether exercise-induced inflammation and lipid peroxidation would be attenuated to a similar extent by antioxidant supplementation.

Specific Aim 4. Can prior supplementation with vitamins E and C attenuate muscle damage and/or accelerate recovery?

Finally, this study was designed to help us elucidate whether specific markers of muscle damage and repair, such as plasma concentrations of creatine kinase and lactate dehydrogenase, as well as measures of muscle fatigue (force recovery) are modulated in response to antioxidant supplementation.

We believe that the idea that oxidative stress is generated in vivo and can be ameliorated by antioxidant supplementation is an important area for research. Beneficial effects of antioxidants in an oxidative stress model such as endurance exercise could be the basis for future studies exploring other human conditions that are associated with oxidative stress such as cardiovascular disease, stroke and diabetes.

LITERATURE REVIEW

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 (44). Enhanced ability to use fat as a fuel through increased free fatty acid transport and metabolism (β oxidation) (45) contributes to the glycogen sparing effect observed in endurance-trained individuals. The glycogen sparing effect of training decreases lactic acid production and thus hydrogen ion (H^+) production. Muscle fatigue results primarily from decreases in cell pH (44). As the trained individual catabolizes less glycogen, there is a reduced flux through the glycolytic pathway and therefore less lactic acid and associated H^+ is produced (45). Fatigue is thus delayed, primarily through effects on cell pH (44).

Changes in the isomer of lactate dehydrogenase enzyme (LDH), the enzyme responsible for the conversion of pyruvate to lactate (45) also contributes to a decrease in production of H^+ with training and subsequent resistance to fatigue. Prior to training, the muscle isomer, LDHm, is predominant, but with endurance training, this enzyme is converted to the cardiac isomer, LDHc, increasing the reaction in the direction of lactate + H^+ $\rightarrow$ pyruvate, enhancing pyruvate uptake into the mitochondria (45), decreasing H^+ production and delaying time to fatigue.

A number of other adaptations to endurance training have been identified. Maximal oxygen consumption (VO_{2max}) can be increased with training; however, steady state oxygen consumption (VO_2) during submaximal

work is not generally affected (45). Endurance training an increase in the size and number of skeletal muscle mitochondria (46), resulting in increased mitochondrial respiratory control, potentially decreasing electron leakage and ROS production. Increased capillary density in the trained muscle allows more nutrients to be delivered to the tissues and increased waste removal (45). Increased capillary density also contributes to decreased blood pressure in the active muscle, facilitating increased O_2 delivery and extraction of waste products.

There is growing evidence that another adaptation to endurance exercise is an up-regulation of the body's antioxidant defense systems. Elevated basal levels of the antioxidant enzymes, superoxide dismutase, glutathione peroxidase, catalase (11) and inducible heme oxygenase (47) have been reported in endurance trained individuals compared with untrained controls. In addition, plasma levels of antioxidant molecules such as vitamin E (13), vitamin C (7, 48) and uric acid (6, 35, 49) are increased in response to exercise. Some investigations have revealed that endurance trained individuals are also more likely to have increased free radical scavenging capacity (6, 49). 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 (marker of muscle damage) following exercise training (46). Taken together, these adaptations to endurance exercise contribute to a delayed time to fatigue, a reduction in muscle damage and an increased endurance capacity.

OXIDATIVE STRESS IN HUMANS

Oxidative stress has been defined as an imbalance between reactive oxygen/reactive nitrogen species production and antioxidant defenses (23). This imbalance can be caused by an increased rate of reactive species production and/or decreased antioxidant protection (50). Damage associated with oxidative stress (e.g. lipid peroxidation, DNA and protein oxidation) is generally referred to as oxidative damage. The generation of oxidative stress in humans, and its reversal by antioxidants has been difficult to demonstrate. However, in the past few years, the use of F_2-IsoPs, a by-product of free radical-mediated arachidonic acid oxidation and thus a marker of in vivo lipid peroxidation, has grown in acceptance. Importantly, studies have shown that supplementation with antioxidants can decrease F_2-isoprostane levels (51). Our laboratory has been investigating the relationship between oxidative stress and human vitamin E requirements. If vitamin E acts as an antioxidant and is destroyed as a result of lipid peroxidation, then vitamin E requirements should increase in response to oxidative stress.

ENDURANCE EXERCISE INCREASES OXIDATIVE STRESS

Despite the many known health benefits of exercise (52) including cardiovascular fitness, maintenance of lean body mass, blood glucose control, positive effects on the lipid profile, and an increased antioxidant defense system, there is a wide body of evidence suggesting that exercise results in oxidative stress. This phenomenon is often referred to as the "paradox of exercise". Vigorous exercise results in increased lipid peroxidation (1), DNA damage (14) and protein oxidation (53). Reactive oxygen species (ROS) leaking from the mitochondria during exercise are considered a main source of oxidative stress (23). Other potential sources of sources of ROS during

exercise include enhanced purine oxidation, damage to iron-containing proteins, disruption of Ca^{2+} homeostasis (24), neutrophil activation (8) and autoxidation of catecholamines (3).

During endurance exercise, runners have higher indices of lipid peroxidation compared to their resting values (1). They also have a faster plasma disappearance rate of vitamin E during exercise (1), suggesting that the runners are under oxidative stress.

OXIDANT PRODUCTION AND THE MITOCHONDRIA

During the resting state, the human body produces 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 (23). Under normal conditions, approximately 1-3% of electrons escape from the mitochondria, resulting in formation of superoxide (23). During endurance exercise there is a 10 to 20-fold increase in whole body oxygen consumption and oxygen uptake in the active skeletal muscle increases 100 to 200-fold (23). It is, therefore, not surprising that increased oxygen utilization during exercise results in increased ROS production (43).

MITOCHONDRIAL ELECTRON TRANSPORT CHAIN

The mitochondrial electron transport chain is not entirely efficient as there is an approximate 1-3% leakage of electrons from the electron transport chain (23). 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 (54). The electron transport chain is embedded within the inner membrane of the mitochondria. It is the primary cellular energy source as it is the site of oxidative phosphorylation and ATP production.

The electron transport chain is composed of four protein complexes; complexes I, III and IV are proton pump locations. In complex I, NADH is oxidized to NAD^+, coenzyme Q (CoQ, also known as ubiquinone), is the electron (e^-) acceptor. CoQ is reduced to the semiquinone radical, which can in turn be reduced by a second e^- to form ubiquinol ($CoQH_2$) (23). Complex II involves the oxidation of $FADH_2$ to FAD and also uses coenzyme Q as its e^- acceptor. Ubiquinol delivers e^- to complex III and finally, e^- are transferred to complex IV by cytochrome C. Complex IV (also known as cytochrome oxidase) is the intended site for the transfer of two e^- (and two protons) to molecular oxygen to form H_2O. During any of these transfers, e^- may inadvertently be transferred to molecular oxygen, rather than to their intended receptor molecule (55). Semiquinone is the most likely to donate an e^- to molecular oxygen (55). Regardless of the mechanism of transfer, when an e^- is transferred to molecular oxygen, the superoxide anion ($O_2^{\bullet-}$) is formed. If $O_2^{\bullet-}$ 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 (56). Although H_2O_2 is relatively non-reactive, it can be cytotoxic at high concentrations e.g. 10-100 μM range (23), as it can oxidize specific keto-acids such as pyruvate and can inactivate certain enzymes such as glyceraldehyde-3-phosphate dehydrogenase. More importantly, H_2O_2 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, H_2O_2 reacts to form the highly reactive hydoxyl radical ($^{\bullet}OH$) (see below). Thus, H_2O_2 can lead to cell damage at a distance relatively far from the original source of reactive oxygen species.

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 (57). Bound iron is non-reactive, but there also exists a small pool of low molecular weight iron that is thought to participate in redox cycling. Unbound iron can react with hydrogen peroxide in the Fenton Reaction (23) to form a hydoxyl radical ($^\bullet$OH).

$$H_2O_2 + Fe^{2+} \rightarrow Fe\ (III) + OH^- + {}^\bullet OH \quad k_2 = 76\ M^{-1}\ s^{-1}$$
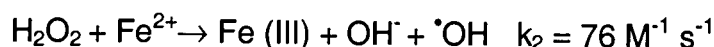
$^\bullet$OH is the most reactive ROS and has the potential to damage DNA, lipid membranes and proteins (23).

Another reaction by which iron and hydrogen peroxide can form hydroxyl radicals is the iron-catalyzed Haber-Weiss (58) reaction:

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

$$Fe^{2+} + H_2O_2 \rightarrow OH^- + {}^\bullet OH + Fe\ (III)\ (23)$$

$$\text{Net: } O_2^{\bullet-} + H_2O_2 \rightarrow O_2 + {}^\bullet OH + OH^- \qquad \text{(Haber-Weiss reaction)}$$
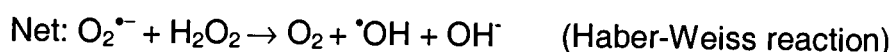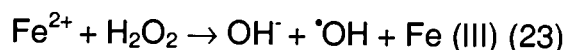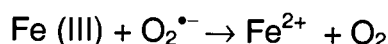
The amount of H_2O_2 accumulating in vivo is minimized by the enzyme catalase.

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

$$\text{compound I} + H_2O_2 \rightarrow \text{catalase-Fe (III)} + H_2O + O_2\ (23)$$
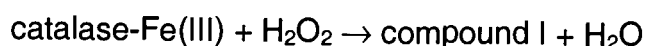
REDOX-ACTIVE METALS AND EXERCISE

Although exercise could result in free radical damage by a number of mechanisms, low molecular weight, redox active iron has been the most studied. According to Jenkins et al. (57), exercise increases the availability of redox active iron. Furthermore, blood pH decreases in response to exercise due to the accumulation of lactate and H^+ (44) and this low pH helps to maintain iron ions in solution (57) making them more readily reactive. During

distance running, intravascular hemolysis (mechanical trauma to erythrocytes) occurs at foot strike as evidenced by increased plasma free hemoglobin (59). The extent of hemolysis increases with increased race distance (60). Damaged erythrocytes provide a potential source of redox active iron in the plasma (57).

Exercise-induced muscle damage results in the release of relatively large amounts of myoglobin, a heme containing protein and another potential source of redox active iron (24, 61). 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 (62, 63). The resulting Ca^{2+} overload may induce Ca^{2+}-activated proteases and phospholipases that can destroy proteins and break down membranes (62), potentially mobilizing redox active iron in the process. Further evidence supporting the theory that exercise increases the prevalence of redox active iron is the appearance of transition metals in sweat (57) and the occurrence of gastrointestinal blood loss during endurance exercise (64).

In healthy people, there are a number of defense mechanisms to mitigate oxidative damage, but they are not 100% efficient (23). It may be that this inefficiency leads to minute amounts of free radical catalyzed cell damage, which over time may impair cell function. This inefficiency may be exacerbated by the increased O_2 utilization associated with exercise. The consequence of such damage may be an increase in available free iron that could then move freely into the cytosol, amplifying oxidative damage.

POTENTIAL ROLE OF XANTHINE OXIDASE

The 10 to 20-fold increase in whole body oxygen consumption and 100 to 200-fold increase in oxygen uptake in the active skeletal muscle during

endurance exercise (23) reflect an increased energy requirement. This exercise associated increase in energy demand may exceed the capacity of the mitochondria to produce ATP, resulting in an accumulation of ADP. One alternative route of ATP production is the cytosolic adenylate kinase system. This mechanism involves the conversion of 2 ADP molecules to 1 AMP + 1 ATP and is catalyzed by the enzyme adenylate kinase (43). 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 (43). 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 (43). Increased calcium may lead to increased activation of calcium-activated proteases such as calmodulin and calpain (23, 43). Both of these proteases catalyze the cleavage of a peptide from XDH (23) 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 $O_2^{\bullet -}$. Thus, ROS production is increased as a by-product of increased energy demands.

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 (56). 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 e^- acceptor for
this reaction to occur. Once exercise ceases, 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 cellular accumulation of
$O_2^{\bullet-}$ along with an increase in plasma uric acid (65).

LIPID PEROXIDATION

Phospholipids are integral components of cell membranes and are
essential for membrane fluidity and transport. Phospholipids generally contain

Figure 1. Lipid Peroxidation

Adapted from Burton & Traber Annu Rev Nutr 10: 357-382; 1990
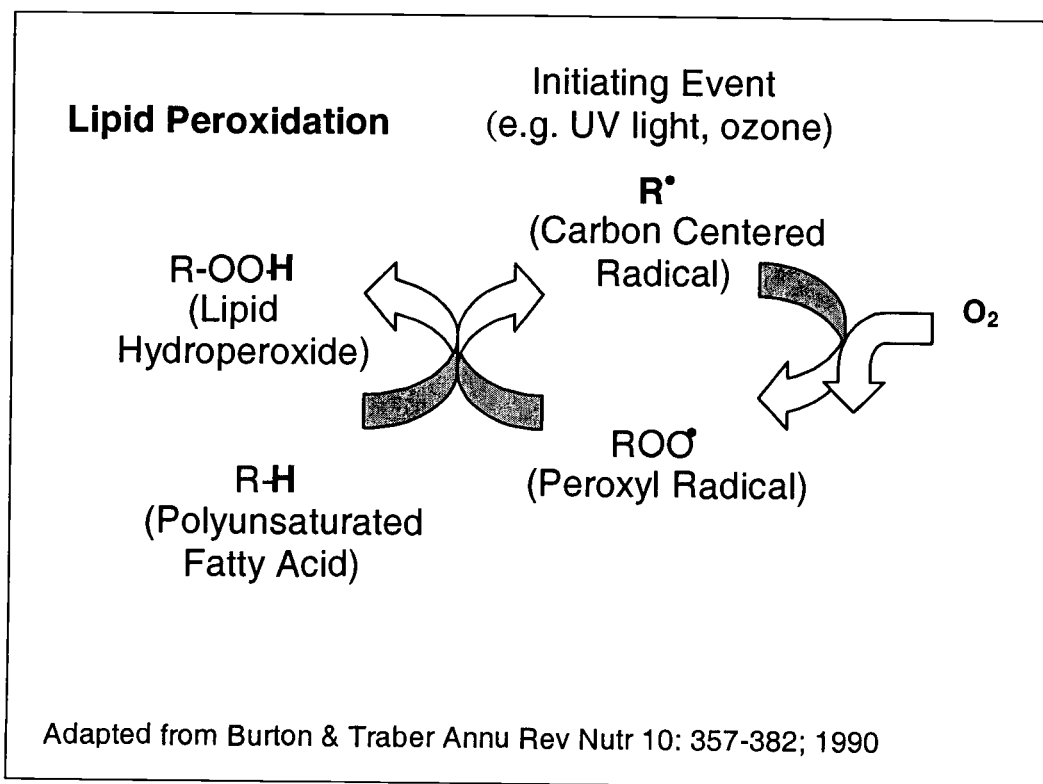
multiple polyunsaturated fatty acids (PUFAs) (fatty acids containing two or more double bonds). These PUFAs are most susceptible to lipid peroxidation because of their chemical structure, which includes bis-allylic hydrogens. These hydrogens are located on a carbon that has double bonds on either side, e.g. $-CH=CH-CH_2-CH=CH-$, making the bonds weaker and thus more susceptible to abstraction. Oxidizability of PUFAs increases proportionally with increases in the number of double bonds (66). A brief outline of lipid peroxidation is shown in **Figure 1**.

Lipid peroxidation is a chain reaction initiated by abstraction of a hydrogen from a carbon forming a carbon-centered radical ($R^\bullet$). The $R^\bullet$ undergoes a conformational change to form the more stable conjugated diene structure (23). In an aerobic environment, the most likely fate of $R^\bullet$ is reaction with molecular oxygen to form a peroxyl radical ($ROO^\bullet$) (23). $ROO^\bullet$ can readily abstract a bis-allylic hydrogen of a PUFA, creating both a lipid hydroperoxide (R-OO-H) and a $R^\bullet$, thus perpetuating a chain reaction. Termination may occur when two radicals react with one another (58), forming an inactive dimer: $R^\bullet + R^\bullet = R - R$; non-radical antioxidants, such as α-tocopherol, may limit the chain reaction.

Damaging effects of lipid peroxidation include impairment of membrane fluidity, disruption of membrane-bound proteins and disruption of active transport of molecules and ions across cell membranes (67). Additionally, lipid peroxidation products may initiate gene transcription or apoptosis, stimulate the immune response, cause inflammation, initiate fibrosis, or inactivate enzymes (68).

MARKERS OF LIPID PEROXIDATION

The rates of free radical reactions are extremely rapid, making it impractical to measure ROS directly (69). Therefore a number of indirect markers have been developed to assess oxidative damage. Previous methods used to assess lipid peroxidation such as the thiobarbituric acid reactive substances (TBARS) assay, have lacked sensitivity/specificity and have been deemed unreliable (70). Differences in the reliability of assays used to assess oxidative stress may account for much of the study variability to date.

Malondialdehyde

Malondialdehyde, a polyunsaturated fatty acid oxidation product (71) has been the most commonly used marker for lipid peroxidation in exercise studies and is often measured indirectly by TBARS. In this colorimetric assay, malondialdehyde reacts with thiobarbituric acid to generate a colored conjugate that can then be measured with a spectrophotometer. The major problems with this assay are that most TBARS detected are generated during sample preparation and the assay is non-specific in that thiobarbituric acid can react with compounds other than malondialdehyde (23).

More recently, high pressure liquid chromatography (HPLC) has been used to increase sensitivity of the MDA assay (71). First, TBA-MDA conjugates are separated from non-specific TBA forms by HPLC followed by detection of TBA-MDA conjugates by UV or fluorescence (71). As this method does not involve the acid hydrolysis step found in the conventional TBARS assay, any MDA bound to protein would be left undetected, leading to an underestimation of MDA (71).

Conjugated Dienes

PUFA oxidation, as discussed above, is accompanied by the formation of conjugated dienes 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 (23). A more useful conjugated dienes assay is to assess the susceptibility of low-density lipoproteins (LDL) to oxidation in vitro (23). However, LDL can be oxidized ex vivo during the long centrifugation time required for isolation.

F_2-Isoprostanes

F_2-IsoPs are unique, prostaglandin-like compounds produced by the non-enzymatic free-radical catalyzed oxidation of arachidonic acid (72). Arachidonic acid (20:4 n-6) is a long chain PUFA and is a precursor for prostaglandin synthesis (54). F_2-IsoPs are structurally distinct from the prostaglandins (73). Unlike the prostaglandins, they are formed while arachidonic acid is still esterified to the phospholipid (74). F_2-IsoPs formation follows that of lipid peroxidation described previously. Initiation occurs with abstraction of a hydrogen atom from arachidonic acid by a free radical forming an arachidonyl R$^{\bullet}$ (74). Reaction of the arachidonyl R$^{\bullet}$ with molecular oxygen, forms a peroxyl radical, 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-IsoPs (74). Of these isomers, 8-epi-$PGF_{2\alpha}$ formation is favored (72, 75); it is also the compound most commonly measured in humans and it has been chemically synthesized (72).

F_2-IsoPs are a sensitive and reliable measure of in vivo lipid peroxidation (73). They are chemically stable, specific end-products of the cyclooxygenase independent free-radical catalyzed oxidation of arachidonic acid (72). Additional advantages of this biomarker are that they remain stable frozen at -70° C for up to 6 months, have a lower limit of detection in the picogram range, and physiological levels are not effected by dietary lipid levels (75, 76). Detectable levels of F_2-IsoPs have been found in all normal animal and human biological fluids and tissues tested (74) and normal plasma levels for humans have been defined (73). This has allowed for the comparison of normal controls with test subjects and as such, F_2-IsoPs have been shown to be elevated in the plasma of subjects with known oxidative stress, such as smokers and patients with cystic fibrosis, asthma and Alzheimer's disease (51, 77-79).

F_2-IsoPs have demonstrated pro-atherogenic biological activity, including vasoconstriction, opposition to nitric oxide and activation of platelet aggregation (75, 76, 80) and they are known to recruit pro-atherogenic monocytes and induce monocyte adhesion.

Most (81-83), but not all (84), studies of high doses of vitamin E on steady state levels of F_2-IsoPs in healthy, non-smoking adults have concluded no effect of the antioxidant. Combinations of antioxidants have provided more positive findings. Upritchard et al. (85) reported that 111 mg/day vitamin E and 1.24 mg/day carotenoids for 11 w reduced plasma F_2-IsoPs by 15%. Supplementation with vitamins E and/or C for a longer duration (~500 mg/d and/or 400IU/day respectively for 8 w) similarly attenuated urinary excretion of F_2-IsoPs (86). Ide et al. (87) reported that vitamin E and C supplementation led to a decrease in urinary excretion of F_2-IsoPs in men, but not in women.

Studies investigating the ability of antioxidants to attenuate F_2-IsoPs in models of known oxidative stress have generated mixed results. Vitamin C alone and in combination with vitamin E, but not vitamin E alone, decreased urinary F_2-isoprostane excretion in chronic cigarette smokers (88). More recently, neither vitamin C alone or together with vitamin E and lipoic acid for 8 w reduced plasma F_2-IsoPs in normal weight smokers (89) but the treatment group had relatively low levels (45 pg/ml), especially compared to the placebo group (58 pg/ml). In a small study of diabetics, treatment for 3 w with *vitamin C* had no effect on plasma F_2-isoprostane levels in persons with diabetes despite relatively high levels (90-100 pg/ml) (90) while in a much larger study, treatment with *vitamin E* for 2 w led to a *37% reduction* in urinary F_2-isoprostane excretion in persons with diabetes in addition to a 43% reduction in excretion of the platelet activation marker 11-dehydro-thromboxaneB$_2$ (91).

ANTIOXIDANTS

Considerations when assessing efficacy of antioxidants to attenuate lipid peroxidation include the type of antioxidant, the oxidant stress model and the duration of supplementation.

Vitamin E

Vitamin E is a fat-soluble micronutrient and as such, it is absorbed with fat via micelles at the intestine, incorporated into chylomicrons and transported to the liver where it is repackaged into lipoproteins by the α-tocopherol transfer protein (α-TTP) or excreted in the bile (26). The term vitamin E refers to the group of eight molecules including four

Figure 2. Vitamin E Structures

Brigelius-Flohe & Traber, FASEB J,13:1145-55;1999
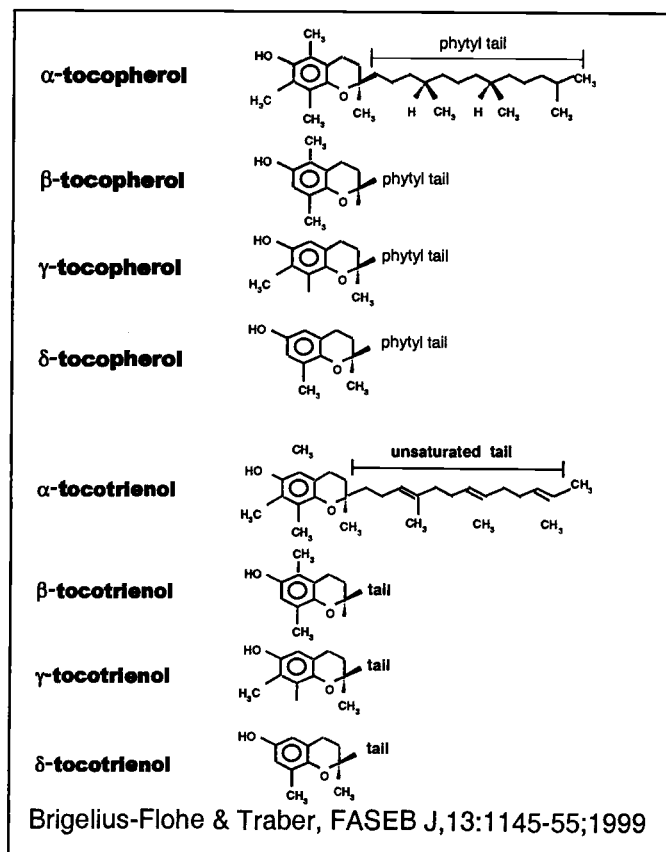
tocopherols, α, β, γ, δ and four tocotrienols, α, β, γ, δ (92)(**Figure 2**). The four tocopherols share a common saturated phytyl tail, but differ in the number of methyl groups on the chromanol ring. The tocotrienols differ from the tocopherols in that they have an unsaturated tail. Of these eight naturally occurring forms, α-tocopherol has the most antioxidant activity and is the most prevalent type found in nature (92). 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 due to differences at the three chiral centers of the phytyl tail (93)(**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,

Figure 3. Synthetic Isomers of Vitamin E

Brigelius-Flohe & Traber, FASEB J,13:1145-55;1999
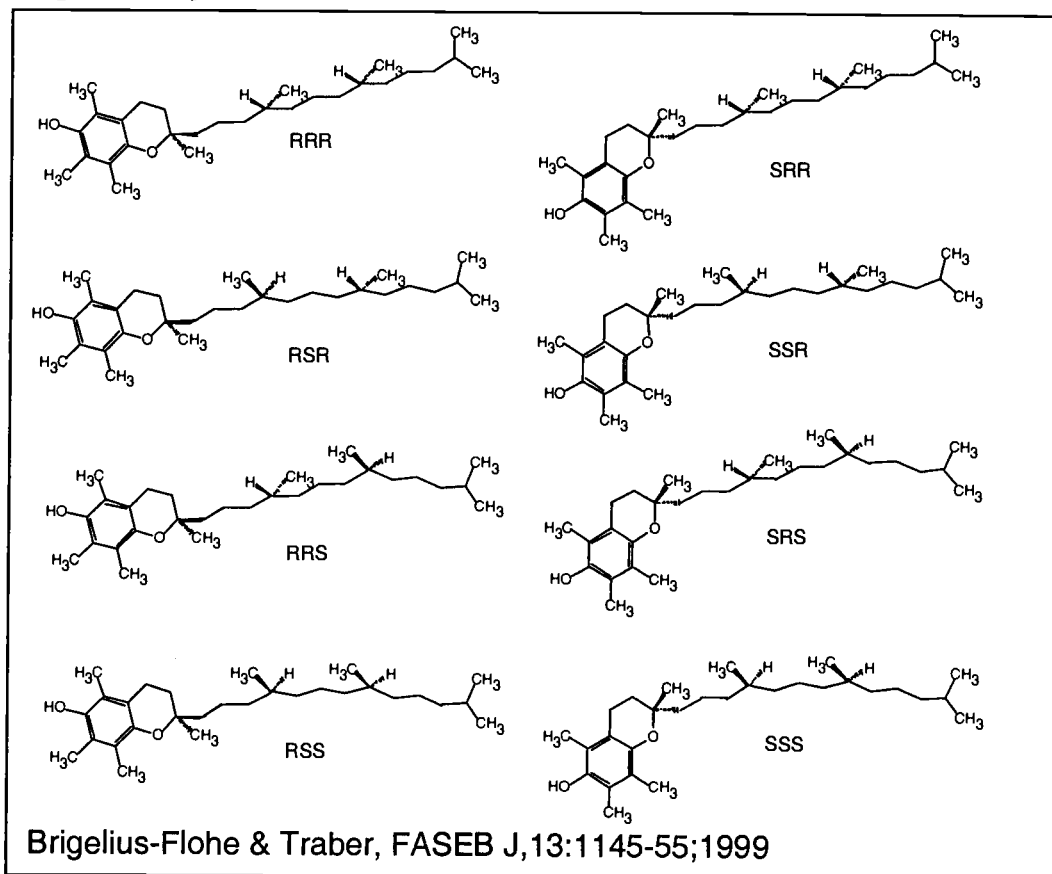
sunflower seeds and whole grains (26, 94, 95). Although the Recommended

Dietary Allowance for vitamin E is only 15 mg/day (94), larger doses of vitamin

E are popular in the United States in the form of dietary supplements (26).

Supplements are typically sold as either acetate or succinate esters

(93), since esterification prevents oxidation, extending shelf life (94). α-

Tocopheryl esters are hydrolyzed and absorbed in the gut with similar

efficiencies (26). While RRR-α-tocopherol and all-rac-α-tocopherol are

absorbed at the intestine and carried to the liver via chylomicrons in a non-

discriminate manner, RRR-α-tocopherol has twice the biological activity of the

synthetic form (94) due to the preferential incorporation of *RRR*-α-tocopherol into very low-density lipoproteins (VLDL) by α-TTP in the liver (93, 96). Consequently, double the proportion of *RRR*-α-tocopherol as compared to all rac is delivered to the tissues by lipoproteins (93).

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, carnitine and neurotransmitters (94). Vitamin C is an essential vitamin because humans lack the enzyme, L-gulonolactone oxidase, required to synthesize ascorbate from glucose (97). Vitamin C is abundant in fruits and vegetables; some of the best sources include asparagus, papaya, oranges, and strawberries (98).

The term vitamin C refers to both the reduced (ascorbic acid) and, the oxidized (dehydroascorbic acid, DHA) vitamin forms (94). The biological functions of ascorbic acid have been defined by its ability to provide reducing equivalents for critical reactions (94). 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 (27). 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* (97).

Levine et al. (99) have demonstrated that leukocyte ascorbic acid levels reach saturation at plasma levels of 50-60 μM. Therefore, they postulated that in order to test for a treatment effect of vitamin C, subjects would need to consume <100 mg vitamin C per day from foods (99). Control of ascorbic acid intake should therefore be a consideration in the design of studies

investigating the efficacy of vitamin C supplementation to prevent oxidative stress.

Uric Acid

Uric acid, a primary end product of purine metabolism and a water-soluble antioxidant (23), increases in response to aerobic exercise (13, 49, 100). This increase may be explained by enhanced purine oxidation with exercise (13, 49, 100), as increased energy requirements characteristic of vigorous exercise upregulate various metabolic pathways including adenylate cyclase (43, 49)(or myokinase in muscle)(101). This enzyme is responsible for production of an ATP and an AMP from 2 ADP (101). While the ATP is used for energy, the AMP is degraded to uric acid (102). During intensive exercise such as an ultramarathon, the enzyme responsible for the conversion of xanthine to uric acid is likely xanthine oxidase, which generates the superoxide radical as a by-product (102). Concurrent increases in plasma ascorbic and uric acids may reflect enhanced antioxidant defenses in response to the oxidative stress of the endurance exercise. These increases in water-soluble plasma antioxidants parallel increases in antioxidant enzymes (11) in response to exercise.

PLASMA ANTIOXIDANTS IN EXERCISE

Antioxidant Activity of α-Tocopherol

Vitamin E (α-tocopherol) is a potent peroxyl scavenger and acts to

protect PUFA against lipid peroxidation (26). α-Tocopherol can quench

peroxyl radicals by donating a H•, reducing the peroxyl radical to a lipid

hydroperoxide (**Figure 4**). The reaction of a peroxyl radical with α-tocopherol

occurs 1000 times faster than the reaction of the peroxyl radical with a PUFA

Figure 4. Antioxidant Function of Vitamin E

Adapted from Burton & Traber Annu.Rev.Nutr. 10: 357-382; 1990
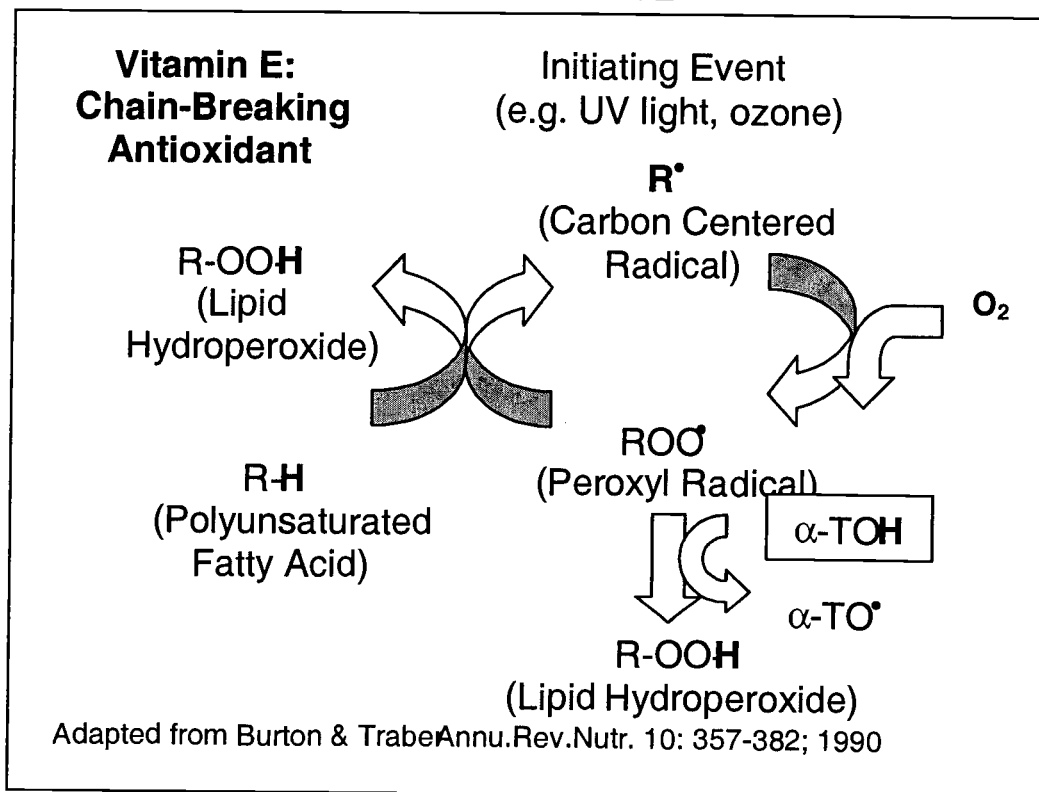
(94). This reaction, however, does not terminate the chain reaction because a

α-tocopheroxyl radical is formed in this process. However, the α-tocopheroxyl

radical is less reactive than the peroxyl radical (58). Possible fates of α-

tocopheroxyl radicals include: the radical can be further oxidized to a quinone

(two electron oxidation), the radical can react with a PUFA to form a peroxyl

radical (pro-oxidant activity), it 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 radical can be reduced back to its active form by another antioxidant, such as ascorbate.

A few of the studies investigating the effects of vitamin E supplementation on endurance running have reported increases in plasma α-tocopherol concentration in both supplemented and placebo groups following exercise (13, 40, 103) but most failed to report post-exercise plasma α-tocopherol concentrations (31, 32, 104-106). A single study reported no change in α-tocopherol concentrations with exercise (107). Only one of these studies reported both absolute α-tocopherol concentrations and α-tocopherol/lipid concentrations (103) and they observed differential responses in the antioxidant and placebo groups that depended on correction for fluctuations in lipid levels. An increase in total plasma α-tocopherol in response to endurance exercise may be due to increased output of α-tocopherol from the liver, perhaps as a result of increased VLDL production, but may also be an oxidative stress-dependent response.

Although some researchers have looked at the effects of vitamin E supplementation on athletic performance or endurance (103, 108) and lipid peroxidation (35, 36, 109), ours was the first to use vitamin E biokinetics to evaluate oxidative stress (1). 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 (96). 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 endurance exercise.

Antioxidant Activity of Ascorbic Acid

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 (28). Not

only does ascorbate prevent lipid peroxidation, it also spares other

antioxidants such as α-tocopherol and urate (27). In response to exercise-

induced oxidative stress, ascorbic acid levels may change in response to

direct oxidation (antioxidant function) or indirect oxidation (regeneration of

other antioxidants). The response of plasma ascorbic acid to vigorous

exercise has been reported previously: increased levels (1, 7, 13, 48, 107,

110), no change (31, 111, 112) or a reduction (113) post-exercise. It has been

suggested that exercise-related increases in cortisol secretion promote efflux

of ascorbic acid from the adrenal gland (114, 115) and/or the mobilization of

ascorbic acid from other tissue sites such as leukocytes or erythrocytes (48).

Some (111, 112, 116), but not all (37) recent studies have demonstrated an

attenuation of the exercise related increase in circulating cortisol with vitamin

C supplementation.

The effects of moderate exercise on plasma vitamin E and C levels

were examined by Viguie et al. (48), studying subjects exercising at 65%

VO_{2max} 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, but they did report an increase in plasma ascorbic acid levels.

ASSESSMENT OF LIPID PEROXIDATION IN ENDURANCE EXERCISE

Malondialdehyde (MDA)/TBARS

Malondialdehyde (MDA) as measured by HPLC or as TBARS have been the most commonly used marker of lipid peroxidation in human exercise trials (71). As discussed previously, the lack of sensitivity/specificity of the assays used to detect this marker of lipid peroxidation helps to explain the conflicting findings arising from exercise studies using these markers.

Maughan et al. (12) studied physically active subjects during a 45 minute downhill run on a treadmill. Downhill running, an eccentric motion, causes more muscle damage than running on a level surface or running uphill (61). 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 muscle damage marker (46), suggesting that muscle damage induced oxidative stress rather than a systemic response. Maughan et al. (12) concluded that there is an association between ROS and the loss of membrane integrity responsible for the release of creatine kinase and other muscle-derived enzymes (12).

Several 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 conflicting results. Marzatico et al. (11) reported increases in MDA indicating that the exercise bout resulted in lipid peroxidation, while Duthie et al. (7) reported no increases in MDA levels in athletes after a similar run.

Goodman et al. (117), also studying a half-marathon run, reported no changes in MDA levels after the data had been corrected for plasma volume changes, but 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 consequence of muscle damage. 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 the portion of the muscle taken in the biopsy did not include the damaged portion (118) or the assays were too insensitive to detect the changes.

Inayama et al. (119) 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 (119). These are surprising results considering that the subjects were only moderately trained, inexperienced distance runners. In agreement with our results from endurance exercise (1), Kanter et al. (9) demonstrated a 77% increase in MDA levels after an 80 km run.

F_2-ISOPROSTANES

Our findings that F_2-IsoPs increase in response to endurance exercise (1) contribute to the accumulating body of evidence that endurance exercise generates lipid peroxidation to an extent that it causes oxidative damage. Muscle damage caused by the resumption of weight bearing activity following

space flight increased urinary excretion of F_2-IsoPs, implicating oxidative stress as a cause of muscle damage (120).

Few other studies have investigated the response of F_2-IsoPs to exercise. Mori et al. (121) trained non-insulin dependent diabetics 30 minutes per day at 55-65% VO_{2max} on a cycle ergometer 3 times per week for 8 w. They reported no increase in *urinary* F_2-IsoPs in response to the moderate exercise training protocol suggesting that the exercise was not strenuous enough to elicit an oxidative stress response. More recently, Nieman et al. (37) reported a significant increase in both lipid hydroperoxides and F_2-IsoPs following an ultramarathon run. Similarly, increases in lipid hydroperoxides and F_2-IsoPs were observed following a non-aerobic muscle damaging protocol (41).

CONJUGATED DIENES

In some cases, exercise-induced oxidative stress has been studied based on the susceptibility of low-density lipoproteins (LDL) to oxidation in vitro. Using LDL obtained from subjects who completed a half-marathon, Case et al. (122) reported an *increase* in lag time of LDL oxidation (protective effect). Conversely, using LDL from subjects after a full marathon Liu et al. (49) reported a *decrease* in LDL lag time. Marzatico et al. (11) 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 differences in analytical technique.

Case et al. (122) conducted one of the few studies involving women, and while two studies in male distance runners yielded no change in conjugated dienes (7, 11). Their group reported an increase in lag time of

conjugated diene formation. Results suggest that in trained female runners, exercise reduces the susceptibility of LDL to oxidation (122). While these studies have involved half-marathon races, Liu et al. (49) reported an increase in oxidizability of LDL in male runners following a full marathon. The question of whether it was the longer distance or gender that caused the differences 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, has antioxidant activity (123, 124). In support of this hypothesis, we have preliminary data demonstrating that F_2-isopsrostanes did not increase in females following a 1 1/2 hour treadmill run to volitional exhaustion at 75% VO_{2max} (125).

Recently, two studies involving both genders addressed the issue of LDL susceptibility to oxidation and again results were mixed. Wetzstein et al. (126) reported an increase in the susceptibility of LDL to oxidation (decrease in lag time) in response to thirty minutes of treadmill running. These results were confounded by the fact that the subject population consisted of both sedentary and trained individuals grouped together. Study findings were further confounded by the fact that the sedentary individuals exercised at a lower *relative* intensity than the trained subjects, such that both intensity AND training status effected outcomes. While both males and females participated in this study, sample size was too small to evaluate whether there were any gender dependent differences. In the most comprehensive study on this subject to date, Shern-Brewer et al. (127) examined the susceptibility of LDL to oxidation in both sedentary and trained individuals in response to an *acute* bout (VO_{2max} test) of exercise. Overall, there was no difference in lag time between the sedentary and the trained subjects. When the groups were split

by gender, there was still no difference in lag time in the female population. The male cohort, however, exhibited a significantly shorter lag time in response to an acute bout of exercise in the sedentary group than in the exercise group. This study has two main implications. First, it offers strong evidence that the effects of exercise on the susceptibility to LDL oxidation may be gender dependent. Secondly, it suggests that training, at least in males, confers some protection against LDL oxidation. However, since this exhaustive exercise bout was only 10-15 minutes in duration, the findings cannot be extrapolated to endurance exercise.

INHIBITION OF EXERCISE-INDUCED LIPID PEROXIDATION BY ANTIOXIDANT SUPPLEMENTATION

Results from studies investigating protection of supplementation with vitamins E and C alone or in combination against exercise-induced lipid peroxidation have been inconclusive: reduced lipid peroxidation (29-35), increased lipid peroxidation (32, 41) or no effect (36-40).

Vitamin E Supplementation

Taken together, the few studies examining the effects of vitamin E supplementation on exercise-induced lipid peroxidation demonstrate a trend for protection by vitamin E (35, 36, 109, 110, 128, 129). However, Meydani et al. (31) reported that 7 w of supplementation with 800 IU dl-α-tocopherol had no effect on urinary TBARS in the 72 hours following a 45 minute downhill run. Itoh et al. (129) on the other hand, reported that 4 w of supplementation with 1200 IU α-tocopherol attenuated the increase in plasma TBARS following six consecutive days of running (~50 min/day) compared to the placebo group. Importantly, supplementation with vitamin E attenuated increases in markers of exercise-induced muscle damage (creatine kinase and lactate

dehydrogenase) (129). Similarly, Sumida et al. (35) reported that 4 w of supplementation with 300 mg d-α-tocopherol inhibited serum MDA formation after a cycle ergometer test to volitional exhaustion. Three w daily supplementation with 300 IU dl-α-tocopherol reduced LDL susceptibility to oxidation following 1 hour of cycling at 70%VO_{2max} (128) and LDL from subjects supplemented with only 13.5 mg/day d-α-tocopherol for 3 w had a 17% lower susceptibility to oxidation following a marathon run than LDL from the placebo group (110). Most recently, Sacheck et al. (32) reported differential effects of supplementation with 1000 IU RRR-α-tocopherol for 12 w prior to a 45 minute downhill run in young and elderly men. Vitamin E attenuated the increase in plasma F_2-IsoPs in the elderly men at 24 h post-exercise, but not at 72 h post exercise. In the young men, supplementation prevented any increases in plasma F_2-IsoPs, but the group initially had higher levels at baseline than the placebo group making interpretation of the results difficult. Furthermore, MDA levels were attenuated at 72 h post-exercise with supplementation in the young men, but they were actually increased 24 and 72 h post-exercise in the elderly men supplemented with vitamin E (32). Since F_2-IsoPs were not measured immediately post-exercise, comparison with other studies in which F_2-IsoPs peaked at post-exercise (1, 2), is not possible.

Vitamin C Supplementation

Vasankari et al. (39) reported that 2000 mg vitamin C had no effect on the exercise-induced increase in serum conjugated diene concentrations. However, upon completion of the exercise, conjugated diene concentrations decreased faster in the supplemented group. The results of a study by Sanchez-Quesada et al. (33) were equally inconclusive. They reported that just one dose of 1000 mg ascorbic acid ingested 30 minutes prior to a 4 hour

run resulted in a decrease in LDL susceptibility to oxidation however, TBARS remained unchanged in both the supplemented and placebo groups. Conversely, Ashton et al. (30) found that a single 1000 mg dose of ascorbic acid consumed 2 h prior to a maximal exercise bout prevented exercise-induced increases in MDA, lipid hydroperoxides and electron spin resonance signal intensity that were observed in the same subjects in a prior exercise bout without supplementation. Alessio et al. (29) also reported that supplementation with 1000 mg ascorbic acid prevented exercise-induced increases in TBARS observed in the placebo group in response to 30 minutes of running at 80%VO_{2max}. Nieman et al. (37) reported no effect of 1500 mg/day ascorbic acid for 1 w prior to an ultramarathon on exercise-induced increases in plasma F_2-IsoPs or lipid hydroperoxides. Childs et al. (41) reported that 1 w supplementation with 12.5 mg/kg body weight ascorbic acid and 10 mg/kg body weight N-acetyl-cysteine (NAC) actually exacerbated exercise-induced increases in both F_2-IsoPs and lipid hydroperoxides 2 days post-exercise, suggesting a *pro-oxidant* effect. These varying results from studies investigating the effects of vitamin C supplementation on exercise clearly call for more research.

Co-Supplementation with Vitamins E and C

A small number of studies have examined the effects of antioxidant combinations on exercise-induced oxidative stress (13, 34, 36, 108, 110). These studies have also yielded inconsistent results. Compared to the placebo group, exercise-induced increases in breath pentane and plasma MDA were attenuated by supplementation with 600 mg α-tocopherol, 1000 mg ascorbic acid and 30 mg β-carotene for 6 w prior to a 30 min run (36). Pfeiffer et al. (108) supplemented recruits with 300 mg α-tocopherol, 500 mg ascorbic

acid, 20,000 IU β–carotene, 100 μg selenium and 30 mg zinc during 14 days of winter altitude training. They reported no effect of supplementation on exercise-induced increases in urinary markers of oxidative stress: TBARS, 8-OHdG or HNE, but *plasma* lipid hydroperoxides increased only in the placebo group, suggestive of protection (108). Inexplicably, runners in both treatment groups, those supplemented with 300 mg α-tocopherol and 200 mg ascorbic acid *or* placebos for 4.5 w prior to a marathon exhibited a *decrease* in plasma TBARS (13). Therefore, the exercise protocol itself, but not antioxidants, appeared to be protective against oxidative damage. Antioxidant supplementation did attenuate muscle damage markers post-race (13), however.

There are a number of explanations for the inconsistent findings with regard to antioxidant protection in exercise. In addition to differences in the modes, duration, and intensity of exercise, 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 (42).

In the present study, we supplemented runners with the antioxidant vitamins E and C daily for 6 w. We chose doses that had demonstrated beneficial effects in other studies (130, 131). We supplemented runners with placebos or 300 mg vitamin E and 500 mg vitamin C twice a day (total of 1000 mg) as these vitamin doses decrease the susceptibility of LDL to ex vivo oxidation (130, 131).

EXERCISE-INDUCED DNA DAMAGE

Several studies have demonstrated exercise-induced lipid peroxidation in response to various modes of exercise, including endurance running (1, 13). Of late, there has been growing interest in exercise-induced DNA damage due to its potential involvement in various disease states. There is speculation that oxidatively damaged DNA is involved in cancer development and an association between oxidative damage to mitochondrial DNA and age-related degenerative diseases has been postulated (132). Past studies have primarily used the 8-hydroxydeoxyguanosine (8-OHdG) assay (133-135) to assess DNA damage, but this method has been criticized for its susceptibility to artifact formation and the large amount of DNA required for analysis (136). More recently, the comet assay (also known as the single cell gel electrophoresis assay) has come into favor due to its greater simplicity, sensitivity and stability (136). The comet assay detects DNA single strand breaks, incomplete excision repair sites and alkali-labile sites (136, 137). Using the comet assay technique, various forms of exercise: high intensity, short duration (138-140); moderate intensity and moderate duration (17); and moderate intensity endurance exercise (15, 19); have all been demonstrated to result in the same relative amounts of DNA damage. Furthermore, exercise-induced DNA damage has been observed in recreationally active individuals (17, 138, 139), trained athletes (15, 19, 140) and sedentary individuals (140), with some evidence that untrained individuals experience greater damage than trained subjects (140).

The purpose of the present study was to determine whether DNA damage is generated in trained women and men during a 50 km ultramarathon run and if so, to assess the extent to which DNA damage could be inhibited by

6 w prior supplementation with the antioxidant vitamins E and C. Previous studies of exercise-induced DNA damage have been generally inconsistent. Both Niess et al. (140) and Hartmann et al. (138) reported an increase in comet tail moment 24 h following a maximal oxygen consumption test on a treadmill (average run time 18.4 ± 1.7 min). Similarly, Hartmann et al. (141) in a study involving only 3 subjects, reported a peak in DNA damage 24 h following a 45 minute run. Niess et al. (17) studied runners following a half marathon (21 km, average run time 93 min), also reporting a peak in comet tail length at 24 h post race, calling into question whether the duration of exercise influences the extent or time course of DNA damage. However, Hartmann et al. (15) reported an increase 24 h after a mini-triathlon, but DNA damage peaked later, at 72 h post-race. The longer duration of the triathlon, 2.5 h, may explain the shift in the peak of DNA damage. One other study has examined DNA damage in endurance exercisers. Tsai et al. (19) used the comet assay to study response to a 42 km run (average run time 3 h). They reported an increase in DNA damage 24 h post exercise that persisted through day 7.

As recently reviewed by Moller and Loft (142), a number of studies have used the comet assay to investigate the ability of antioxidant supplementation to attenuate basal DNA damage in sedentary subjects. Supplementation in general had little effect on DNA damage, however Moller and Loft (142) suggested that a protective effect of antioxidants may only be seen in the presence of an oxidative stressor such as exhaustive exercise. To test whether vitamin E supplementation could inhibit exercise-induced DNA damage using the comet assay, Hartmann et al. (138) studied five men in response to a total of four consecutive maximal exercise bouts (average run

time 15 min: maximum running time 18.4 minutes). The four trials included: no supplement (test I), a multivitamin supplement (test II), 800 mg vitamin E 2 h before and 22 h following exercise (test III) or 1200 mg vitamin E for 14 days prior to the exercise bout (test IV). While all three supplement regimens inhibited DNA damage, the 1200 mg vitamin E for 14 days prior to the exercise bout (test IV) appeared to have the greatest effect, leading the investigators to conclude that vitamin E prevents exercise-induced DNA damage. However, the order of the tests was not randomized, making it impossible to determine if the protective effects of vitamin E resulted from the supplementation, or due to a training effect.

EXERCISE-INDUCED INFLAMMATION

Endurance and/or damaging exercise elicits a stress response analogous to the acute phase immune response (25). A local response to a stressor such as tissue injury or ROS, stimulates production of a group of low molecular weight regulatory proteins, called cytokines, which regulate the inflammatory cascade (25, 143, 144). In parallel to the local response, there is a systemic inflammatory response characterized by fever, leukocytosis, production of acute phase proteins and transfer of extracellular iron to intracellular stores (25). Initially, the pro-inflammatory cytokines, tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) are produced, these in turn stimulate the production of interleukin-6 (IL6) an inflammation-responsive cytokine (143). IL-6, considered the primary mediator of the acute phase reaction, is responsible for stimulating production of acute phase proteins including the hepatocyte derived C-reactive protein (CRP) and restricting the extent of the inflammatory response by stimulating production of anti-inflammatory cytokines such as interleukin-1 receptor antagonist (IL-1ra) (144,

145). While cytokines recruit neutrophils, monocytes and lymphocytes to the site of inflammation, these cells, in turn, produce ROS and proteolytic enzymes in order to clear and repair damaged tissue (25, 143, 145, 146).

It has been postulated that ROS may stimulate cytokine production in response to exercise because ROS induce cytokine production from various cell types including skeletal muscle (106). Therefore it has also been hypothesized that antioxidant supplementation may attenuate the stress response to exercise (106). The few studies examining the effects of antioxidant supplementation on exercise-stimulated cytokine production have generated mixed results. Vitamin C supplementation attenuated the increase in cortisol (37, 111) but enhanced the increase in CRP (111). While most studies found no effect of vitamins E or C on the cytokine response to exercise (105, 107, 111, 116), Cannon et al. (147) reported an attenuation of the increase in IL-1β with vitamin E supplementation (400 IU for 48 days) and Vassilakopoulos et al. (106) reported that increases in TNF-α, IL-1β and IL-6 were all prevented with an antioxidant cocktail that included vitamins E, A and C (200 mg, 50,000 IU, and 1000 mg, respectively) for 60 days.

Very few studies have studied the effects of antioxidants on both exercise-induced oxidative stress and inflammation (37, 41). Nieman et al. (37) reported that 1500 mg/day ascorbic acid for 1 w prior to an ultramarathon had no effect on exercise-induced increases in plasma F_2-IsoPs or lipid hydroperoxides. Similarly, the supplementation appeared to have no effect on increases in the inflammatory markers IL-6, IL-1$_{RA}$, IL-10, IL-8, IL-2 and IFN-γ. Using an eccentric arm exercise protocol known to induce muscle damage, Childs et al. (41) studied the effects of 1 w supplementation with 12.5 mg/kg body weight ascorbic acid and 10 mg/kg body weight N-acetyl-cysteine (NAC)

on markers of lipid peroxidation and inflammation. Both F_2-IsoPs and lipid hydroperoxides increased in response to the damaging protocol, but levels reached even higher concentrations in the supplemented group 2 days post-exercise, suggesting a pro-oxidant effect. IL-6 concentrations increased similarly in both groups following exercise, indicating that the supplementation had no effect on inflammation.

OVERVIEW

The purpose of the present study was to determine if prior supplementation with the antioxidant vitamins E and C could protect against the oxidative damage, inflammation and muscle damage generated during endurance exercise. The effects of antioxidant supplementation on the potentially adverse effects of endurance exercise are presented in the following chapters: Chapter 2, exercise-induced DNA damage; Chapter 3, lipid peroxidation and inflammation; Chapter 4, vitamin E kinetics; Chapter 5, muscle fatigue, damage and subsequent recovery. Chapter 6 offers a discussion of the present study and its implications with regard to endurance exercise, the use of antioxidant supplements and directions for future research.

CHAPTER 2: ENDURANCE EXERCISE RESULTS IN DNA DAMAGE AS DETECTED BY THE COMET ASSAY

Angela Mastaloudis[1,2], Tian-Wei Yu[2], Robert P. O'Donnell[3,4], Balz Frei[2], Roderick H. Dashwood[2] and Maret G. Traber[1,2,5,6]

Dept. of Exercise and Sport Science[1], Oregon State University, Corvallis, OR; Linus Pauling Institute[2], Corvallis, OR; Hewlett-Packard[3], Corvallis, OR; Department of Statistics[4], Oregon State University, Corvallis, OR; Dept. of Nutrition and Food Management[5], Oregon State University, Corvallis, OR; Dept. of Internal Medicine, University of California Medical Center[6], Davis, CA

ENDURANCE EXERCISE RESULTS IN DNA DAMAGE AS DETECTED BY THE COMET ASSAY

ABSTRACT

To determine if 6 w supplementation with antioxidants could alleviate exercise-induced DNA damage, we studied 21 runners during a 50 km ultramarathon. Subjects were randomly assigned to one of two groups: 1) placebos (PL) or 2) antioxidants (AO) (1000 mg vitamin C and 400 IU RRR-α-tocopheryl acetate). The comet assay was used to assess DNA damage in circulating leukocytes at selected time points: pre-, mid-, and 2 h post-race, and for 6 days post-race. All subjects completed the race; run time 7.1 ± 0.1 h; energy expenditure 5008 ± 80 for women (n = 10) and 6932 ± 206 kcal for men (n = 11). Overall, the % DNA damage increased at mid-race ($p < 0.02$), but returned to baseline by 2 h post-race, indicating that the exercise bout induced non-persistent DNA damage. There was a gender*treatment*time interaction ($p < 0.01$). One day post-race, women taking AO had 62% less DNA damage than women taking PL ($p < 0.0008$). In contrast, there were no statistically significant differences between the two treatment groups of men at any time point. Thus, endurance exercise resulted in DNA damage as shown by the comet assay and AO appeared to enhance recovery in women but not in men.

INTRODUCTION

During the resting state the human body produces reactive oxygen species (ROS) continuously, but in healthy individuals these ROS are produced at levels well within the capacity of the body's antioxidant defense system. However, endurance exercise elicits a 10 to 20-fold increase in whole body oxygen (O_2) consumption and O_2 consumption at the level of the skeletal muscle increases 100 to 200-fold. This increase in O_2 utilization may result in the production of ROS at rates that exceed the body's capacity to detoxify them (67). Potential sources of ROS during exercise include leakage of electrons from the mitochondrial electron transport chain (23), enhanced purine oxidation, damage to iron-containing proteins, and disruption of Ca^{2+} homeostasis (24). Left unchecked, these ROS may cause protein, lipid and/or DNA damage.

Several studies have demonstrated exercise-induced lipid peroxidation in response to various modes of exercise, including endurance running (1, 13). Recently, there has been growing interest in exercise-induced DNA damage due to its potential involvement in various disease states. There is speculation that oxidatively damaged DNA is involved in cancer development and an association between oxidative damage to mitochondrial DNA and age-related degenerative diseases has been postulated (132). Past studies have primarily used the 8-hydroxydeoxyguanosine (8-OHdG) assay (133-135) to assess DNA damage, but this method has been criticized for its susceptibility to artifact formation and the large amount of DNA required for analysis (136). More recently, the comet assay (also known as the single cell gel electrophoresis assay (SCG Assay)) has come into favor due to its greater

simplicity, sensitivity and stability (136). The comet assay detects single strand DNA breaks, incomplete excision repair sites and alkali-labile sites (136, 137). Using the comet assay technique, various forms of exercise: high intensity, short duration (138-140); moderate intensity, moderate duration (17); and moderate intensity endurance exercise (15, 19) have all been demonstrated to produce the same relative amounts of DNA damage. Furthermore, exercise-induced DNA damage has been observed in recreationally active individuals (17, 138, 139), trained athletes (15, 19, 140) and untrained individuals (140), with some evidence that untrained individuals experience greater damage than trained subjects (140).

The purpose of the present study was to determine whether DNA damage is generated in recreationally-trained women and men during a 50 km ultramarathon run and if so, to assess the extent to which DNA damage could be alleviated by 6 w prior supplementation with vitamins E and C.

EXPERIMENTAL PROCEDURES
STUDY DESIGN

Subjects

The protocol for this study was approved by the Oregon State University Institutional Review Board for the Protection of Human Subjects. Runners (11 women and 11 men) were recruited from the pool of participants in the McDonald Forest Ultramarathon Race, an annual event that takes place in Corvallis, Oregon. The 50 km (32 mile) ultramarathon consists of a trail run over rugged terrain, with a total elevation gain and loss of >12,000 ft. The physical characteristics of the subjects at baseline are presented in **Table 1**. Men differed from women with regard to all anthropometric measures except

age. Women in the antioxidant group weighed less than women in the placebo group (p< 0.05) while men in the antioxidant group were slightly taller than men in the placebo group (p< 0.05). Within each sex, VO_{2max} and % body fat did not differ among treatment groups. Training regime (estimated by average running mileage per week) did not differ between treatment groups or sexes (**Table 1**).

Criteria for Subject Participation

Inclusion criteria for participation in the study included non-smoking status, age 18-60 y, and a VO_{2max} classified as excellent fitness by Powers and Howley (45). Potential participants were excluded based on antioxidant supplement use (e.g. vitamin C, vitamin E, selenium or carotenoids), abnormal cholesterol, triglyceride or glucose levels, other supplement use (performance enhancing, or herbal type products), vegetarian or other restrictive dietary requirements, pregnancy or suspected pregnancy, and chronic upper respiratory infections.

Pre-screening

Baseline information including: $VO_{2\ submax}$, body composition assessment, a general health screening questionnaire and a blood chemistry were collected.

Blood Chemistry

An initial blood screening was used to identify subjects with abnormal cholesterol ($\geq$ 7.8 mmol/L (300 mg/dl)), triglyceride ($\geq$ 3.8 mmol/L (300 mg/dl)) or fasting blood glucose levels ($\geq$ 7.8 mmol/L (140 mg/dl)). Plasma vitamin E and vitamin C levels were also measured in order to identify any supplement users.

Table 1. Subject Characteristics.

Characteristic	Females		Males	
	AO (N=6)	PL (N=5)	AO (N=6)	PL (N=5)
Age (yrs)	41 ± 6	35 ± 4	41 ± 6	39 ± 8
Height (cm)*	165 ± 2	167 ± 3	182 ± 2^a	176 ± 2^a
Weight (kg)*	57 ± 1^b	63 ± 2^b	81 ± 4	75 ± 5
%body fat*	21 ± 3	22 ± 3	16 ± 3	13 ± 2
LBM (kg)*	45 ± 2	49 ± 2	68 ± 2	64 ± 3
BMI*	21 ± 0.4	23 ± 0.9	25 ± 1.0	24 ± 1.3
VO_{2max} (ml/kg/min)*	55 ± 2	54 ± 4	58 ± 2	62 ± 1
Training (mi/wk)	33 ± 5	26 ± 3	22 ± 5	28 ± 5

Values are means $\pm$ SE. LBM, lean body mass; BMI, body mass index; VO_{2max}, maximal oxygen consumption.
[a]male ao vs male pl $p < 0.05$; [b]female ao v female pl $p < 0.05$;
*male vs females $p < 0.05$

Submaximal Oxygen Consumption

In order to establish that subjects were of a similar, marathon level of fitness, maximal oxygen consumption ($VO_{2\ max}$) was extrapolated from a submaximal oxygen consumption test by taking advantage of the linear relationship between heart rate and workload oxygen consumption using a progressive treadmill run test to achieve 85% age-predicted maximum heart rate. Age predicted maximum heart rate was calculated by the equation: Predicted Heart Rate (bpm) = 220 - age (yrs) (45). Respiratory gases were collected and analyzed using a SensorMedics Metabolic cart (SensorMedics Yorba Linda, California). Heart rate was recorded using a Polar heart rate monitor (Polar Electro Inc Woodbury, NY). The relationship between work rate, oxygen consumption, and heart rate was analyzed using data obtained from the metabolic cart. Based on the criteria presented by Powers and Howley (45), subjects were classified as having excellent fitness and accepted into the study if their VO_{2max} was $\geq$ 47, 45, 44 or 41 $ml \cdot kg^{-1} \cdot min^{-1}$, for men aged 21-29, 30-39, 40-49 and 50-60 years, respectively, or $\geq$ 37, 36, 33 or 32 $ml \cdot kg^{-1} \cdot min^{-1}$, for women aged 21-29, 30-39, 40-49 and 50-60 years, respectively.

3-Day Diet Records

Three-day diet records (two week days and one weekend day), were used to estimate subjects' average daily intake of nutrients including energy, vitamin E and vitamin C intake at baseline, prior to the beginning of the study. Records were analyzed using the Esha Food Processor Program (Salem, OR). Estimated average daily intake was: 2653 $\pm$ 201 kcal, 142 $\pm$ 26 mg vitamin C and 14 $\pm$ 3 mg vitamin E; there were no differences between men and women in energy, dietary vitamin E or C intakes.

Randomization Criteria

Subjects were randomly assigned in a double-blind fashion to one of two treatment groups (placebos, or antioxidants (300 mg RRR-α-tocopheryl acetate and 1000 mg ascorbic acid (500 mg twice daily)). A blocking technique was used to ensure that the treatment group populations were homogeneous with regard to select physical characteristics (148). Blocking factors used in the randomization included age, sex, plasma triglycerides and cholesterol. An unpaired t-test was used to confirm that the randomization was successful in creating homogeneous groups with regard to the blocking factors.

Diet

Subjects were instructed to consume a prescribed diet low in vitamins E and C for the 6 w prior to the race and the six days following the race and then a controlled diet for 2 days: 1 day prior to the race and race day.

All foods for the 2 days of the controlled diet were prepared and provided to the participants in the Metabolic Feeding Unit in the Human Nutrition Research Laboratory at Oregon State University. Subjects were then instructed to resume the vitamin C and E restricted diet for 6 days post race. Both diets consisted of 10-15% protein, 55-65% carbohydrate, and 25-30% fat; the macronutrient breakdown recommended for optimal athletic performance (45, 101).

For the entirety of the study, subjects were provided with energy bars, carbohydrate pastes and carbohydrate drinks low in vitamins E and C to be used for fuel during their training and during the race.

Blood Draws

Blood samples were obtained 1 hour prior to the race (pre-race (0h)), in the middle of the race at kilometer 27 (mid-race (5 h)), 2 hours after race end (2 h post-race (10 h)) and daily for six days following the race (post 1-7 days (24-144 h)), for a total of nine time points. All blood draws were fasting, in the early morning except mid-race, post-race and 2 h post-race.

Blood Samples

Blood was drawn into two 5 cc green-top Vacutainer tubes (containing 143 USP units sodium heparin) and one 5 cc purple-top Vacutainer tube (containing 1 mg/ml EDTA). Aliquots of whole blood were taken immediately for the comet assay analysis. Remaining blood was centrifuged at 2500 x g for ten minutes; plasma was then aliquoted to cryotubes for various assays. Samples were flash frozen in liquid nitrogen and stored at –80°C until time of analysis (within 6 months of collection).

For vitamin C analysis, freshly drawn plasma (50 µL) 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.

Activity Log

For the 6 w prior to the ultramarathon, subjects recorded daily the amount, type, duration and intensity of exercise performed each day. This information was used to identify inter-subject differences in training regimen.

Non-Steroidal Anti-Inflammatory Drugs (NSAIDs)

Subjects were asked to refrain from using NSAIDs including naproxen sodium and ibuprofen throughout the 7 w of the study. As an alternative to these drugs, subjects were allowed to use acetaminophen.

Race Schedule

Body Weight: Subjects were weighed immediately before and after the race to estimate losses in body water.

Foods and Beverages: selected foods and beverages were provided to the subjects at check points along the race course, allowing for quantification of the caloric and nutrient intake of the subjects, and as a means to control vitamin E and vitamin C intake before and during the race.

Heart Rate Monitor: Subjects wore a Polar heart rate monitor (Polar Electro Inc., Woodbury, NY) during the run; average heart rate in conjunction with oxygen consumption levels, measured previously in the lab, were then used to quantify individual energy expenditure during the race.

Physical Activity: Subjects were asked to refrain from physical activity, especially running, for the six days following the ultramarathon in order to investigate the effects of antioxidant supplementation on recovery.

Energy Expenditure Calculation

Energy expenditure was calculated based on average heart rate during the run and the corresponding oxygen consumption (VO_2) multiplied by the time it took each subject to finish the race.

Ave HR (bpm) $\rightarrow VO_2$ (L/min) X 5 = kcal/min X Race Time (min) = Total kcals

Body Mass

Body Mass Index (BMI) = weight in kg/(height in m^2)

% Body Fat

A Bod Pod® (Concord, CA), an apparatus that estimates % body fat based on changes in pressure created by the displacement of air by the body, was used to determine %body fat.

Lean Body Mass (LBM)

LBM (kg) = (100 - %bodyfat) X total body mass (kg)

ASSESSMENT OF DNA DAMAGE

Assessment of DNA damage was carried out using the comet assay according to the method of Anderson et al. (149). Briefly, slides were prepared by adding ~5 µL fresh whole blood, mixed with 60 µL 0.5% low melting point agarose (LMPA) sandwiched between a layer of normal melting point agarose (NMPA) and a layer of 0.5% LMPA on a fully frosted slide. In order to release the DNA, cells were lysed by immersing slides in lysing solution until time of analysis (3-14 days) at 4°C. A longer lysis time at low temperature increases the sensitivity and reproducibility of the comet assay (150), therefore lysis in our experiment was carried out at 4°C for 3-14 days. It has been reported previously that slides may be stored for extended periods of time in cold lysing solution (up to several months) without affecting results if 10% DMSO has been added (151). Lysing solution composition: 10% DMSO (added freshly), 2.5 M NaCl, 100 mM EDTA, disodium salt, 10 mM Tris, 1% Sodium lauryl sarcosinate, 1% Triton X-100 (added freshly); pH 10. DNA was denatured by placing slides in an alkaline bath in an electrophoresis tank for 30 minutes to allow for the unwinding of the DNA and expression of alkali-labile damage. Electrophoresis was carried out with an electric current of 0.8 V/cm applied for 30 min; pH electrophoresis solution > 13. Tris buffer was

added onto slides to neutralize excess alkali after electrophoresis. Finally, DNA was stained by adding 60 µl ethidium bromide (EtBr) (20 µg/ml) to each slide.

Comet measurements were made by image analysis using a fluorescence microscope and the Comet Assay III Software. Images of 50 randomly selected nuclei (25 nuclei from each of two replicate slides) were analyzed from each sample. A representative comet image is shown in **Figure 5**. The comet measurements recorded and subsequently used for analysis were tail length, % DNA in tail, tail moment and the proportion of cells out of 50 with tail moment (tail intensity X tail length) $\geq 0.51 \pm 0.04$ (**Figure 5**). This final parameter is referred to as the "proportion of cells with damage" and is a novel means of assessing comet results.

Figure 5. Comet Image and Measurements.
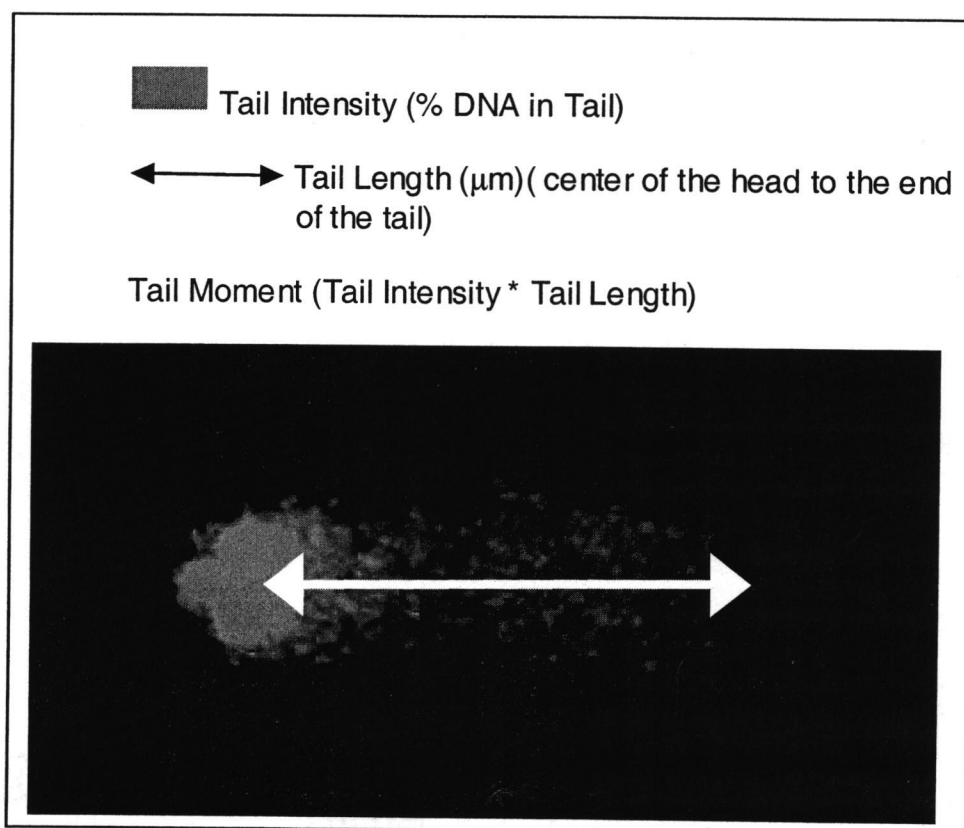
PLASMA ANTIOXIDANTS

Tocopherols were measured using a modified version of the method of Podda et al. (152). α-Tocopherol was determined by paired-ion reversed-phase HPLC coupled with electro-chemical detection. Ascorbic acid was determined by paired-ion reversed-phase HPLC coupled with electro-chemical detection using a modification of the method described by Kutnink et al. (153).

STATISTICAL ANALYSES

The Analysis of Covariance for repeated measures designs was used to assess statistically significant differences of between and within subject effects (154). Data are expressed as the mean ± standard error. Statistics were calculated using The SAS® System (SAS Institute Inc., Cary, NC). In order to adjust for pre-existing differences between individuals prior to the exercise bout, pre-race (time 0 h) levels of DNA damage were used as a covariate in the statistical model (the analysis found the covariate to be statistically significant, $p < 0.03$). One subject was removed from the analyses due to extreme comet values falling greater than two standard deviations above the mean at all timepoints including baseline. An unpaired t-test was used to analyze differences between the sexes with regard to subject characteristics (i.e. age, height, weight).

RESULTS

RACE RESULTS

All 21 subjects completed the race (**Table 2**). Run time averaged 7.1 ± 0.2 h at a pace of 13.7 ± 0.4 min/mile and heart rate (HR) of 146 ± 2 bpm; there were no statistically significant differences in run time, pace or heart rate between the sexes or the treatment groups. Energy expenditure was approximately 2000 kcal greater for men than women, but was not different

Table 2. Race Results Mean ± SE

Parameter	Females AO (N=6)	Females PL (N=5)	Males AO (N=6)	Males PL (N=5)
Run Time (hr)	7.1 ± 0.4	7.3 ± 0.6	6.8 ± 0.4	7.2 ± 0.5
Pace (min/mile)	13.2 ± 0.7	13.6 ± 1.1	12.8 ± 0.8	13.4 ± 1.0
Ave HR (bpm)	147 ± 8	149 ± 5	147 ± 4	140 ± 6
EE (Kcal)*	4997 ± 207	4958 ± 187	6928 ± 558	7006 ± 297
EE (Kcal/min)*	12 ± 1	12 ± 1	17 ± 2	17 ± 1
Energy Intake (Kcal) *	1844 ± 137	2040 ± 221	2530 ± 325	2468 ± 279
Vit E Intake (mg)	3 ± 1	2 ± 1	3 ± 1	4 ± 1
Vit C Intake (mg)	39 ± 14	32 ± 9	28 ± 5	17 ± 5

Values are means ± SE. HR, heart rate; EE, energy expenditure.
* male vs females $p < 0.05$

Table 3. Plasma Antioxidant Levels

Antioxidant	Treatment Group	Baseline	6 Weeks Supplementation
α-Tocopherol (μM)	Supplement	28 ± 2	45 ± 3[+,$]
	Placebo	24 ± 2	28 ± 2
Ascorbic Acid (μM)	Supplement	113 ± 14	121 ± 9[+,$]
	Placebo	93 ± 11	78 ± 9

Values are means ± SE.
[+]$p < 0.0001$ compared to baseline. [$]$p < 0.0007$ compared to PL group.

between treatment groups for each sex. Energy intake was greater in men than women but did not differ between treatment groups.

Vitamin E and vitamin C intakes from foods consumed before, during and for two hours following the race were calculated. Vitamin E intake during the run was nominal; < 5 mg for each group, and did not differ between the treatment groups or the sexes. Similarly, vitamin C intake was low (< 50 mg for most subjects) and did not differ between the treatment groups or the sexes (**Table 2**).

PLASMA ANTIOXIDANTS

At baseline (day 0, prior to supplementation), plasma α-tocopherol levels were similar in the antioxidant supplementation and placebo groups (Table 3). Following 6 w of supplementation, plasma α-tocopherol increased 30% from 28 ± 2 to 45 ± 3 μM in the antioxidant supplement group (p < 0.0001) and were higher than in the placebo group (p < 0.0007); levels in the placebo group were unchanged.

Ascorbic acid levels did not differ between the groups at baseline. After 6 w of supplementation, the antioxidant supplement group had higher plasma ascorbic acid levels, 121 ± 9 μM, than did the placebo group, 78 ± 9 μM (p < 0.0007) (Table 3); levels in the placebo group were unchanged.

DNA DAMAGE

Tail moment is the product of tail length and % DNA in tail; thus tail moment represents both the amount of DNA migrated into the tail and the distance migrated (**Figure 5**). The tail moment is commonly reported (15, 137, 138, 140, 155-159) as a valid marker of single strand DNA breakage. Collins (136) concluded that at low levels of damage the tail length appears to be a more sensitive marker than % DNA in tail. Since our subjects exhibited low

levels of damage, tail moment, which incorporates both the tail length and %DNA in tail was calculated.

The mean % DNA in tail is presented in **Figure 6**. The % DNA in tail are presented here as mean ± standard deviation (SD) to emphasize the large variances that result from a few very large cells skewing the data; data for all other parameters is presented as mean ± SE. No significant interactions were detected. Only a main effect of time reflecting the increase in DNA damage at mid-race was statistically significant.

Figure 6. Percentage DNA in Tail. Shown are the mean ± SD of the % DNA in the tail which increased at mid-race compared to pre-race (ANOVA main effect for Time; $p < 0.05$). There were no statistically significant differences detected between sexes or treatment groups using this metric.
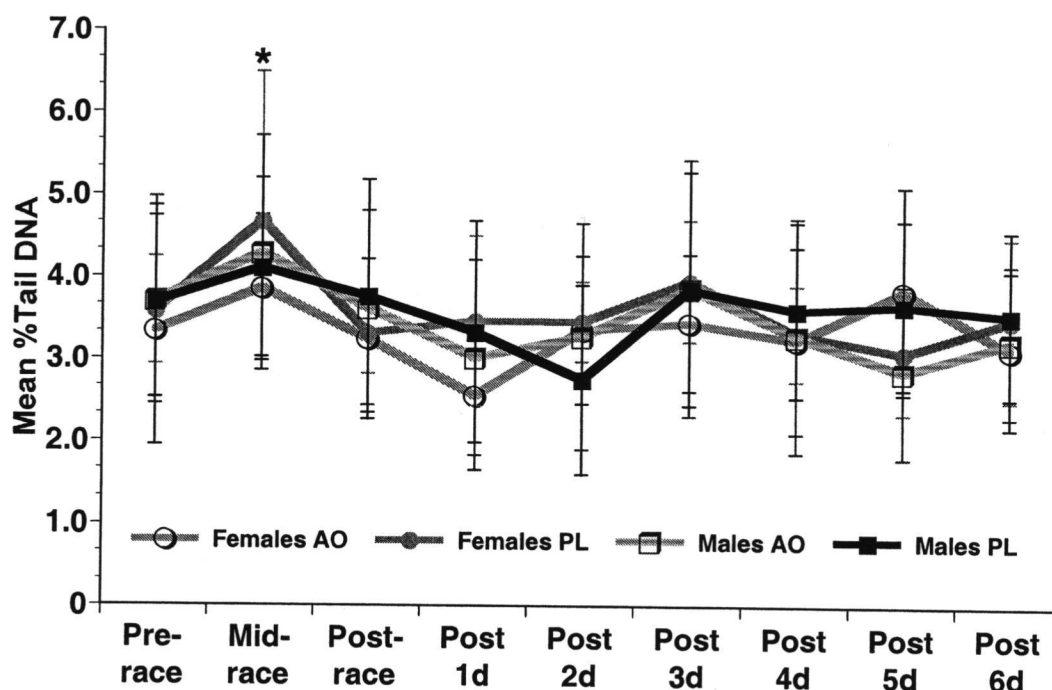
Similarly, the distribution of tail moments and %DNA in tail from a representative subject (**Figures 7A and 7B**) illustrate the various sizes of tail moment and %DNA in tail as well as the skewed distribution of the cell population. It is obvious that a few extremely large comets are responsible for

Figure 7. Distribution of DNA Damage; 50 Individual Cells Imaged in a Representative Subject at Pre-Race. 50 Individual cells ranked by tail moment **(A)**. 50 Individual cells ranked by % DNA in tail **(B)**.
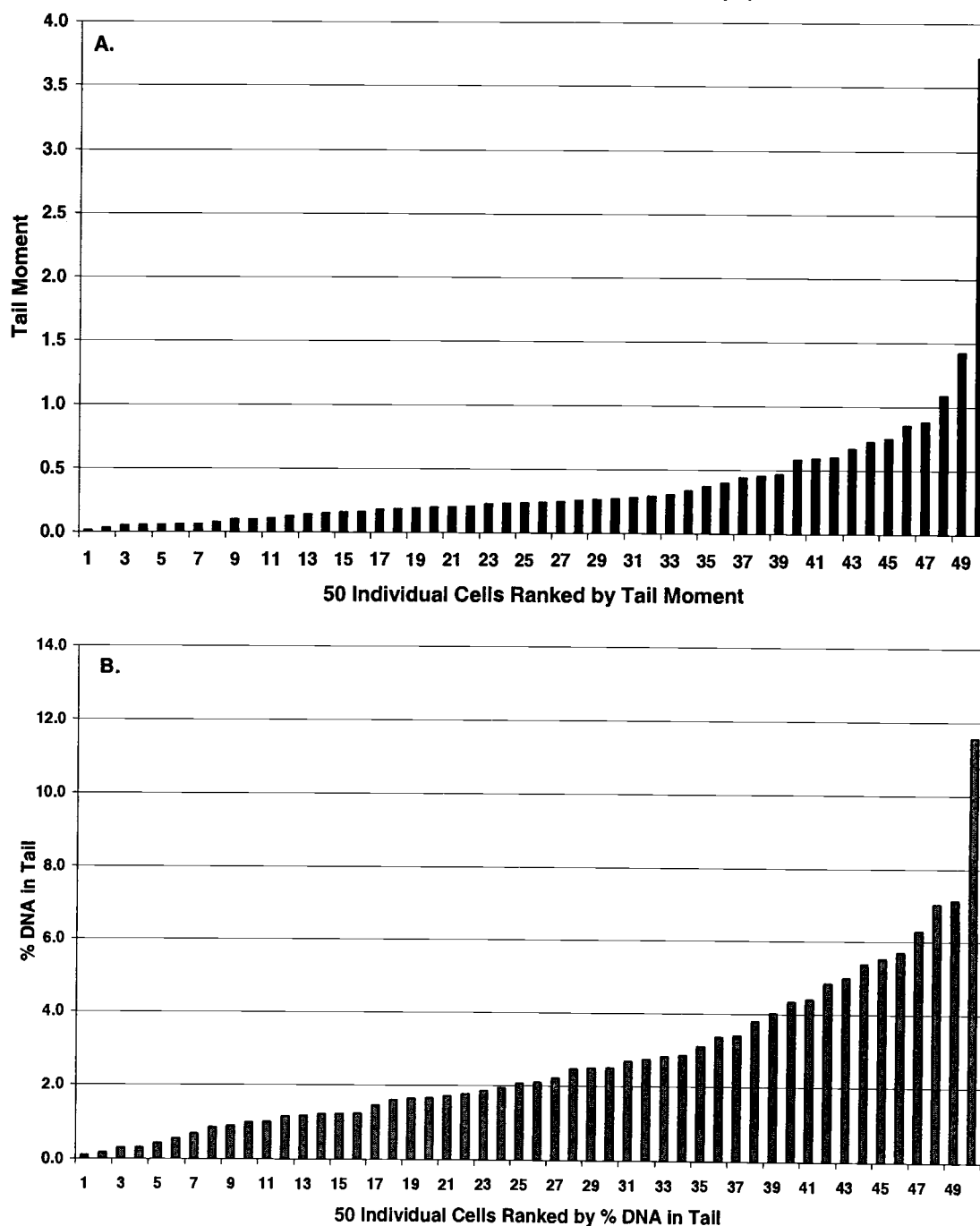
the skewed distribution. Thus neither measure (% DNA in tail or tail moment) met one of the key assumptions of most statistical tests, that of a normal

distribution. The observation of a skewed distribution has been reported by others (15, 141, 155, 157, 160). The lack of a normal distribution in our study was not corrected by standard transformations such as natural log transformation and therefore it necessitated we develop a novel approach to analyze the data.

Our strategy was to calculate the mean tail moment at baseline for all subjects (no damage): mean tail moment = 0.51 ± 0.04. Then, at each time point, the number of cells out of 50 with a tail moment larger than 0.51 (mean tail moment at baseline) was calculated. This value was divided by 50 (total number of cells analyzed per person per time point) to yield the "proportion of cells with DNA damage".

For all subjects, the proportion of cells with DNA damage were found to increase ~10% at mid-race ($p < 0.02$) (**Figure 8**). The proportion of damaged cells was elevated, then decreased, returning to pre-race levels by 2 h post-race. The damage continued to decline such that by 6 days (144 h) following the exercise bout the proportion of cells with damage was 8% lower than pre-race ($p < 0.02$).

With regard to the vitamin E and vitamin C supplementation, women and men responded differently to the treatment over the time course of the study (ANCOVA, Treatment x Gender x Time interaction, $p < 0.01$). The evidence of antioxidant protection was greatest in the women on the day following the race (24 h), such that women in the antioxidant group had 62% fewer cells with damage than women in the placebo group (treatment simple effect, $p < 0.0008$) (**Figure 9A**). Unlike the women, no significant differences between treatment groups were observed in men (**Figure 9B**).

Figure 8. Percent Change From Pre-Race. Shown are the % changes compared to pre-race in the proportion of cells with DNA damage. The proportion of cells with DNA damage was calculated as the proportion of cells out of 50 with tail moment $\geq 0.51 \pm 0.04$. The proportion of cells with damage at mid-race (3 h) increased 10% compared to pre-race ($p < 0.02$). The damage then decreased, such that at 6 days (144 h) following the exercise bout it was significantly lower than pre-race (* $p < 0.02$ compared to pre-race (0 h)).
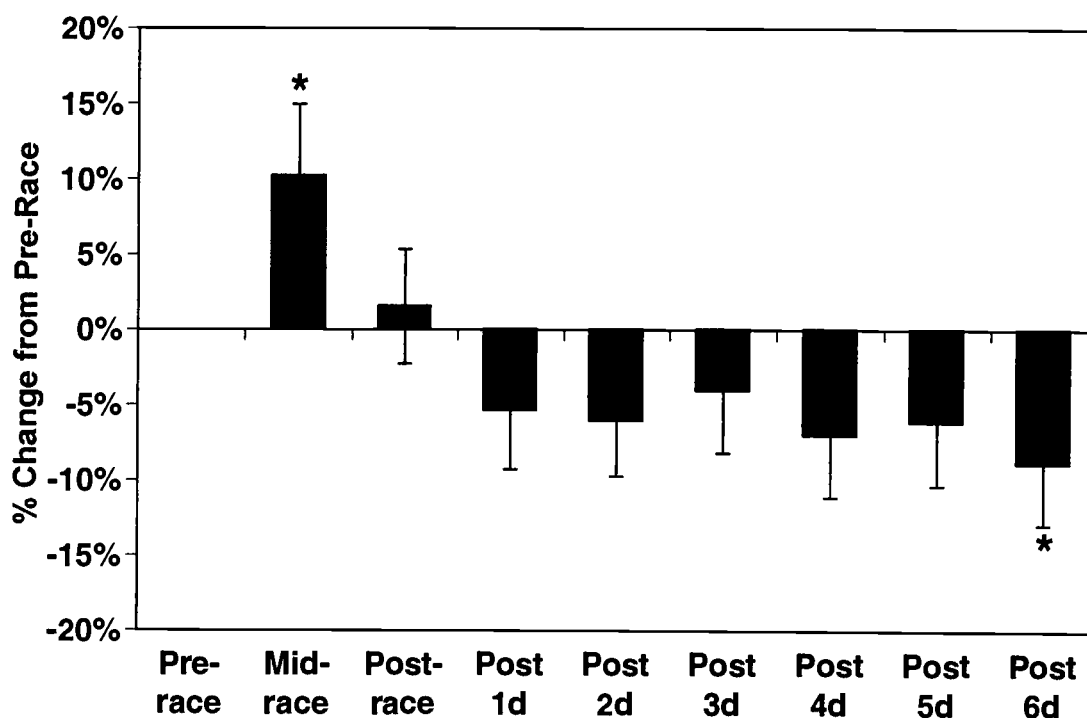
Other sex differences were also observed. On the day following the race (24 h), the proportion of cells with DNA damage in the placebo groups was 42% in the women compared with 26% in men ($p < 0.006$). Similarly in the antioxidant groups, five days (120 h) after the race, the proportion of cells with DNA damage was 40% in women compared with 19% in men ($p < 0.006$).

Figure 9. Proportion of Cells with DNA Damage: Effects of Antioxidant Supplementation. A significant gender X treatment X time interaction for the four groups was observed ($p < 0.01$). Antioxidants were protective in women on the day following the race (# $p < 0.0008$ antioxidant women vs. placebo women) **(A)**. No differences between treatment groups were exhibited in men **(B)**. Data shown have been adjusted for pre-existing differences between individuals by using pre-race (time 0 h) levels of DNA damage as a covariate in the statistical model (covariate, $p < 0.03$).
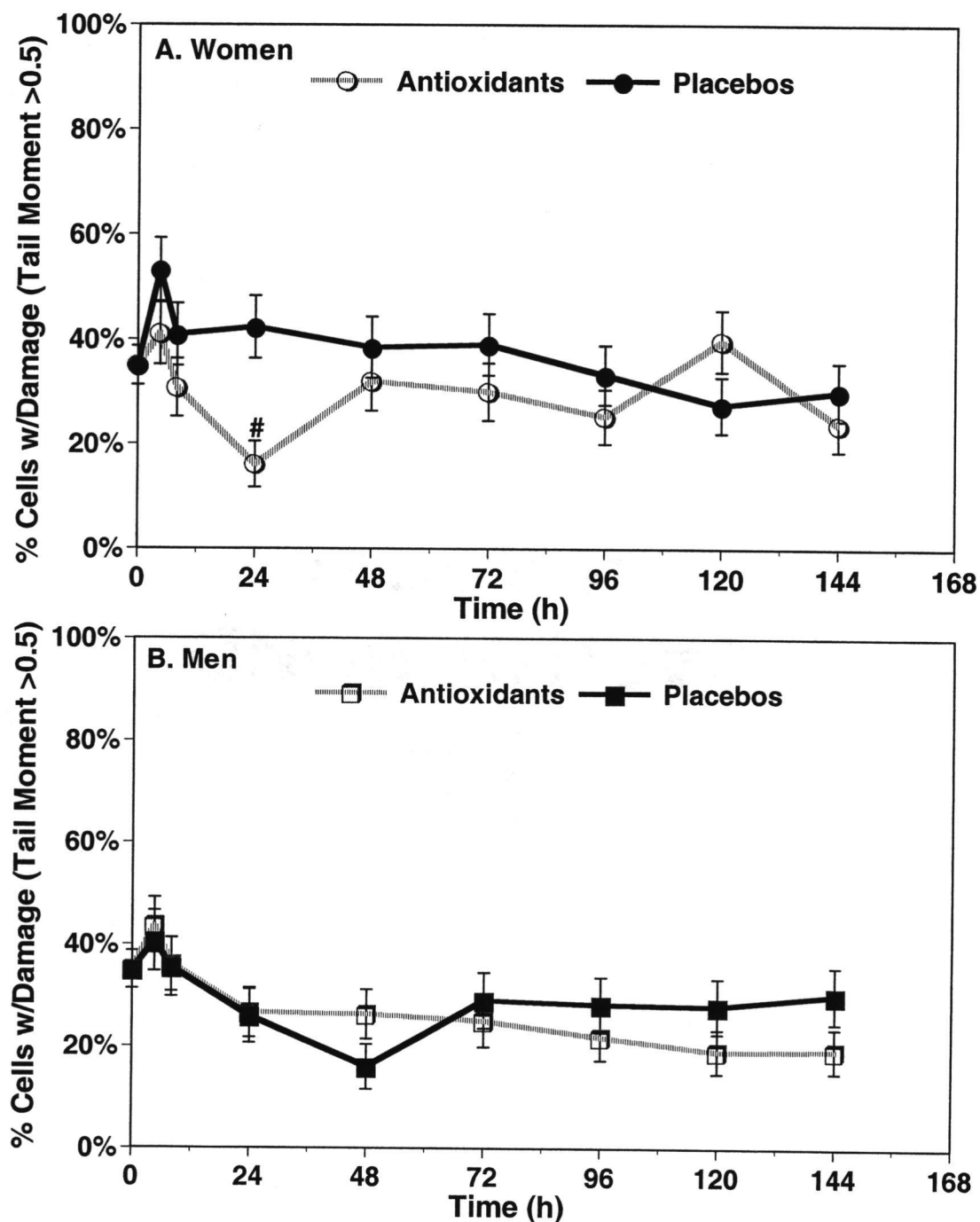
DISCUSSION

Antioxidant supplementation protected women runners by decreasing the proportion of cells with DNA damage on the day following the ultramarathon race (**Figure 9A**). Importantly, men and women responded differently to the supplements over time with men experiencing little benefit from the antioxidants. This finding of sex differences highlights the importance of studying both sexes in exercise studies rather than simply generalizing the findings from studies investigating only men.

On average, subjects exhibited DNA damage by mid-race (**Figure 8**) indicating that the exercise bout was sufficiently strenuous to test the effects of antioxidants on DNA damage. However, the proportion of cells with DNA damage returned to baseline values by the end of the race. Furthermore, by 2 days after the race, the proportion of cells with damage declined below baseline values and decreased to 8% below baseline by 6 days post-race. The precise reason for this apparent decrease is unclear but may have been due to increased repair mechanisms, increased clearance of damaged cells, a redistribution of damaged cells and/or because subjects were no longer exercising.

Regardless of the exercise protocol studied, increases in DNA damage in peripheral human white cells have been reported generating consensus that exercise does indeed induce DNA damage (132). Based on these previous studies, we chose to measure DNA damage in circulating leukocytes. However, it is not possible to determine if the damage observed is representative of damage at the level of the skeletal muscle. One study (133) reported a relationship between DNA damage profiles in muscle cells following 2 w of hypoxia compared to mononuclear blood cells after 3 days of hypoxia.

No conclusions about a relationship between peripheral blood cells and muscle cells can be drawn until a study is conducted measuring both cell types in the same subject.

In previous studies the time courses of exercise-induced DNA damage have been generally inconsistent. Both Niess et al. (140) and Hartmann et al. (138) reported an increase in comet tail moment 24 h following a maximal oxygen consumption test on a treadmill (average run time 18.4 ± 1.7 min). Similarly, Hartmann et al. (141) in a study involving only 3 subjects, reported a peak in DNA damage 24 h following a 45 minute run. Niess et al. (17) studied runners following a half marathon (21 km, average run time 93 min), also reporting a peak in comet tail length at 24 h post race, calling to question whether the duration of exercise influences the extent or time course of DNA damage. However, Hartmann et al. (15) reported an increase 24 h after a mini-triathlon, but DNA damage peaked later, at 72 h post-race. The longer duration of the triathlon, 2.5 h, may explain the shift in the peak of DNA damage.

One other study has examined DNA damage in endurance exercisers. Tsai et al. (19) used the comet assay to study the response to a 42 km run (average run time 3 h). They reported an increase in DNA damage 24 h post exercise that persisted through day 7. The duration of the exercise bout in our study was longer (7 hours with extreme elevation changes), than any previous studies. It may be that the exhaustive nature of our extreme endurance run caused damage greater than in previous studies, and therefore, the damage appeared earlier in the time course. Previously, in runners participating in the Mc Donald Forest Ultramarathon, we found that lipid oxidation was detectable

by mid-race and peaked at race end (1), again suggesting enhanced clearance of oxidation products in response to mountain trail running.

As recently reviewed by Moller and Loft (142), a number of studies have used the comet assay to investigate the ability of antioxidant supplementation to attenuate basal DNA damage in sedentary subjects. Supplementation in general had little effect on DNA damage, however Moller and Loft (142) suggested that a protective effect of antioxidants may only be seen in the presence of an oxidative stressor. Hartmann et al. (138) studied five men in response to a total of four consecutive maximal exercise bouts (ave run time 15 min: maximum running time 18.4 minutes). The four trials included: no supplement (test I), a multivitamin supplement (test II), 800 mg vitamin E 2 h before and 22 h following exercise (test III) or 1200 mg vitamin E for 14 days prior to the exercise bout (test IV). While all three supplement regimens inhibited DNA damage, the 1200 mg vitamin E for 14 days prior to the exercise bout (test IV) appeared to have the greatest effect, leading the investigators to conclude that vitamin E prevents exercise-induced DNA damage. However, the order of the tests was not randomized, making it impossible to determine if the protective effects of vitamin E were the result of the supplementation, or due to a training effect.

We found that despite similar circulating antioxidant levels in both sexes within each treatment group, higher levels of DNA damage were observed in women runners. Specifically, a significant three-way "Treatment x Gender x Time" interaction ($p < 0.01$) was observed, resulting from: 1) a treatment effect of antioxidants compared to placebo in women at 24 h post race; 2) a gender effect in the placebo groups, women had greater DNA-damage compared with men at 2 days post-race ($p < 0.006$); and 3) a gender effect in the antioxidant

groups, women had more DNA-damage compared with men at 5 days post-race (p < 0.006). Previously, Bajpayee et al. (161) reported significantly higher levels of steady state DNA damage in women compared to men using the comet assay. Together these findings suggest that women have higher levels of DNA damage than men, and that women may benefit from antioxidant supplementation.

Exercise-induced oxidative damage may be beneficial by enhancing antioxidant enzyme systems and/or enhancing clearance of oxidized LDL particles (123). Relatively low levels of DNA damage generated by exercise may actually be beneficial by upregulating DNA damage repair enzymes, stimulating the immune response and/or by increasing clearance of damaged cells by inducing apoptosis (162). This concept that low levels of a damaging entity can upregulate protective mechanisms in an organism is known as hormesis (162). In support of this theory, both cohort and case-control epidemiological studies have demonstrated dose-dependent protection of physical activity against overall and site-specific cancer risk (163). Moreover, there is little evidence that the exercise-induced DNA damage is mutagenic (137). In fact, Hartmann et al. (15) reported that despite substantial increases in DNA damage in response to a 2.5 h triathlon competition, no increases in frequency of micronuclei, a sensitive indicator of chromosome damage, were observed.

In general, in our study antioxidant supplementation did not inhibit DNA damage induced by the exhaustive run, but rather impacted post-exercise damage. One potential explanation for the lack of protection by antioxidants is that DNA damage was assessed in circulating leukocytes, which are known to concentrate ascorbic acid. The placebo group had relatively high plasma

ascorbic acid levels (78 ± 9 μM) and Levine et al. (99) has demonstrated that leukocyte ascorbic acid levels reach saturation with plasma levels of 50-60 μM. Saturation levels of ascorbic acid in leukocytes in both the antioxidant group and the placebo group could explain the lack of a treatment effect. For these reasons, Levine et al. (99) postulated that in order to test for a treatment effect of vitamin C, subjects in the placebo group would need to consume less than 100 mg of vitamin C per day from foods. Subjects in the present study were instructed to consume a prescribed diet consisting of foods low in vitamins C and E and vitamin C intake was limited to <50 mg during the race period. However, the high average daily caloric intake required to sustain the excessive energy expenditure of training (2500-3000 kcal/day), likely led to consumption of approximately 100 mg of vitamin C per day despite the relatively small amounts of vitamin C in the low vitamin C foods subjects consumed.

Overall, endurance exercise resulted in DNA damage detected at mid-race using the comet assay, as evidenced by the increases in the proportion of cells with DNA damage using our novel method of calculation. Thus, we conclude that running does induce DNA damage, and that the observation of different effects in males and females is an interesting observation that merits further investigation. The average maximal oxygen consumption of the participants, 59 ml/kg/min, indicates that the subjects were well trained, but not elite athletes, making the results applicable to the general physically active population. Men and women responded differently in this study; therefore, it is critical that future studies investigating the effects of antioxidant supplementation on exercise-induced oxidative damage involve both sexes.

CHAPTER 3: ANTIOXIDANT SUPPLEMENTATION PREVENTS EXERCISE-INDUCED LIPID PEROXIDATION, BUT NOT INFLAMMATION, IN ULTRAMARATHON RUNNERS

Angela Mastaloudis[1,2], Jason D. Morrow[3], Dawn W. Hopkins[1,2], Sridevi Devaraj[4]; and Maret G. Traber[1,2,5]

[1]Department of Exercise and Sport Science, Oregon State University, Corvallis, OR; [2]Linus Pauling Institute, Corvallis, OR; [3]Departments of Medicine and Pharmacology, Vanderbilt University School of Medicine, Nashville, TN; [4]Laboratory for Atherosclerosis and Metabolic Research, Department of Pathology, University of California Medical Center, Sacramento, CA; [5]Department of Nutrition and Food Management, Oregon State University, Corvallis, OR

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Antioxidant Supplementation Prevents Exercise-Induced Lipid Peroxidation, but Not Inflammation, in Ultramarathon Runners

ABSTRACT

To determine if 6 w supplementation with vitamins E and C could alleviate exercise-induced lipid peroxidation and inflammation, we studied 22 runners during a 50 km ultramarathon. Subjects were randomly assigned to one of two groups: 1) placebos (PL) or 2) antioxidants (AO; 1000 mg vitamin C and 300 mg RRR-α-tocopheryl acetate). Blood samples were obtained prior to supplementation (baseline), after 3 w supplementation, 1 h pre-, mid-, post-, 2 h post- and for six days post-race. Plasma α-tocopherol (α-TOH), ascorbic acid (AA), uric acid (UA), F_2-isoprostanes (F_2-IsoPs), tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6) and C-reactive protein (CRP) were measured. With supplementation, plasma α-TOH and AA increased in the AO but not the PL group. Although F_2-IsoP levels were similar between groups at baseline 28 ± 2 (PL) and 27 ± 3 pg/ml (AO), F_2-IsoPs increased during the run *only* in the PL group (41 ± 3 pg/ml). In PL women, F_2-IsoPs were elevated at post-race (p < 0.01), but returned to pre-race concentrations by 2 h post-race. In PL men, F_2-IsoP concentrations were higher at post-, 2 h post and for 1, 2, 3, 4, and 6 days post-race (PL vs. AO group, each p < 0.03). Markers of inflammation were increased dramatically in response to the run regardless of treatment group. Thus, AO supplementation prevented endurance exercise-induced lipid peroxidation but had no effect on inflammatory markers.

INTRODUCTION

Strenuous exercise causes oxidative stress resulting in lipid peroxidation (1, 6-8, 11, 13, 20) and DNA damage (132). Evidence of protein oxidation resulting from exercise-induced oxidative stress is less definitive (20, 22). In response to endurance exercise, oxygen (O_2) consumption increases 10 to 20-fold systemically (164) and as much as 100 to 200-fold at the level of the skeletal muscle (23), resulting in substantially increased mitochondrial electron flux. Reactive oxygen species (ROS) "leaking" from the mitochondria during exercise are considered a main source of oxidative stress (23). Other potential sources of ROS during exercise include enhanced purine oxidation, damage to iron-containing proteins, disruption of Ca^{2+} homeostasis (24) and NADPH oxidase (8). These exercise-induced ROS are also thought to modulate acute phase inflammatory responses (25).

Previously we demonstrated a near two-fold increase in F_2-IsoPs and accelerated disappearance of deuterium-labeled α-tocopherol immediately following a 50 km ultramarathon (1). Therefore, our rationale for the present study is based on this observation of increased lipid peroxidation in endurance runners. Hypothetically, supplementation with the antioxidant vitamins E and C could alleviate exercise-induced lipid peroxidation. However, results from previous studies investigating the protective effects of supplementation with vitamins E and/or C have been inconclusive: inhibition of lipid (29-35), no effect (36-40) and even increased lipid peroxidation (32, 41) in response to exercise following supplementation. Possible reasons for these inconsistencies include: differences in modes, duration, and intensity of exercise as well as variation in the methodologies used to assess lipid

peroxidation. A lack of consensus regarding the effectiveness of antioxidants to inhibit exercise-induced lipid peroxidation and the popularity of antioxidant supplements in the physically active community make this an important area for research (42).

Endurance and/or damaging exercise elicits a stress response analogous to the acute phase immune response (25). Exercise-induced tissue damage and/or increased reactive oxygen species (ROS) production stimulates cytokine production, up-regulating the inflammatory cascade (25, 143, 144). Initially, pro-inflammatory cytokines, TNF-α and IL-1β, are produced, stimulating IL-6 production (143). IL-6, the primary mediator of the acute phase reaction, stimulates production of acute phase proteins, including CRP and restricts the extent of the inflammatory response by enhancing production of anti-inflammatory cytokines (144, 145). Neutrophils, monocytes and lymphocytes recruited to the site of inflammation produce ROS and proteolytic enzymes in order to clear and repair damaged tissue (25, 143, 145, 146).

It has been postulated that, in response to exercise, ROS stimulate cytokine production from various cell types including skeletal muscle (106). Thus, antioxidant supplementation may attenuate this stress response to exercise (106). The few studies examining the effects of antioxidant supplementation on exercise-stimulated cytokine production have generated mixed results. Vitamin C supplementation has been demonstrated to attenuate increases in cortisol (37, 111), but to stimulate increases in CRP (111). Most studies found no effect of vitamins E or C on the cytokine response to exercise (105, 107, 111, 116) but Cannon et al. (147) reported an attenuation of the increase in IL-1β with vitamin E supplementation and

Vassilakopoulos et al. (106) reported that increases in TNF-α, IL-1β and IL-6 were all prevented with an antioxidant cocktail. However, only two studies (37, 41) included a marker of lipid peroxidation, therefore the link between ROS and cytokine production remains unresolved.

The purpose of the present study was to determine whether exercise-induced lipid peroxidation and inflammation could be alleviated by 6 w prior supplementation with vitamins E and C in recreationally trained women and men participating in an ultramarathon run. We believe that this is the first study to investigate the effects of both vitamin E and vitamin C on exercise-induced lipid peroxidation using F_2-IsoPs in addition to measuring inflammatory markers.

EXPERIMENTAL PROCEDURES
HUMAN SUBJECTS

A description of the study design has been published previously (14). Briefly, the protocol for this study was approved by the Oregon State University Institutional Review Board for the Protection of Human Subjects. Runners (11 women and 11 men) were recruited from the pool of participants in a 50 km (32 mile) ultramarathon trail run. All subjects were physically fit, non-competitive athletes. Prior to enrollment into the study, all subjects completed a submaximal oxygen consumption ($VO_{2\ submax}$) test, body composition assessment, a standard blood chemistry screening, a 3-day diet record and a general health-screening questionnaire. For the $VO_{2\ submax}$ test the Bruce Treadmill Protocol was used (165). Subjects ran on a treadmill beginning at a moderate pace, every 3 minutes the grade and intensity were increased until subjects achieved 85% of their age predicted maximum heart

rate (HR). Age predicted maximum HR was calculated by the equation: Predicted Heart Rate (bpm) = 220 - age (yrs) (45). Oxygen consumption and carbon dioxide expiration were measured throughout the test using a SensorMedics Metabolic cart (SensorMedics Yorba Linda, CA). Heart rate was recorded using a Polar HR monitor (Polar Electro Inc. Woodbury, NY). For body composition assessment, a Bod Pod was used to determine % body fat. Three-day diet records (2 week days and 1 weekend day) were used to estimate subjects' average daily intake of vitamins E and C prior to the beginning of the study. Records were analyzed using Esha Food Processor Program (Salem, OR).

The physical characteristics of the subjects have been reported previously (14). Average age 39 ± 2.5 yrs, VO_{2max} 58 ± 1 ml/kg/min and estimated weekly training distance 43 ± 3 km; there were no differences between treatment groups or genders for these characteristics. Men were taller, weighed more and had lower percent body fat than women. AO women weighed slightly less than PL women ($p < 0.05$) while AO men were slightly taller than PL men ($p < 0.05$). Within each gender, VO_{2max} and % body fat did not differ among treatment groups. Estimated average daily nutrient intakes were not different between men and women: 142 ± 26 mg vitamin C and 14 ± 3 mg vitamin E.

CRITERIA FOR SUBJECT PARTICIPATION

Inclusion criteria for participation in the study included non-smoking status, age 18-60 y, and a VO_{2max} classified as excellent fitness by Powers and Howley (45). Potential participants were excluded based on antioxidant supplement use (e.g. vitamin C, vitamin E, selenium or carotenoids), abnormal cholesterol (≥ 7.8 mmol/L (300 mg/dl)), triglyceride (≥ 3.8 mmol/L (300 mg/dl))

or fasting blood glucose levels (≥ 7.8 mmol/L (140 mg/dl)) or other supplement use (performance enhancing, or herbal type products), vegetarian or other restrictive dietary requirements, pregnancy or suspected pregnancy, and chronic upper respiratory infections.

STUDY DESIGN

Randomization to Treatment Group

Subjects were randomly assigned in a double-blind fashion to one of two treatment groups 1) PL (300 mg soybean oil and 1000 mg citric acid (500 mg twice daily) or 2) AO (300 mg *RRR*-α-tocopheryl acetate and 1000 mg ascorbic acid (500 mg twice daily)).

Blood Samples

Blood samples were obtained prior to supplementation (baseline), following three w of supplementation (compliance), 1 hour prior to the race (pre-race, 0h)), in the middle of the race at kilometer 27 (mid-race, ~5 h), immediately post-race, 2 hours after race end (2 h post-race, ~10 h) and daily for six days following the race (post 1-6 days, 24-144 h), for a total of twelve time points. All samples were fasting morning blood draws except mid-, post- and 2h post-race.

Blood was drawn into two 5 cc green-top Vacutainer tubes (containing 143 USP units sodium heparin) and one 5 cc purple-top Vacutainer tube (containing 1 mg/ml EDTA). Blood was centrifuged at 2500 x *g* for ten minutes; plasma was then aliquoted to cryotubes for various assays. Samples were flash frozen in liquid nitrogen and stored at −80°C until time of analysis.

For vitamin C analysis, freshly drawn plasma (50 µL) 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.

Diet

Subjects were instructed to consume a restricted diet low in vitamins E and C for the 6 w prior to the race and the six days following the race (in conjunction with the supplements or placebos) and then a controlled diet for 2 days: 1 day prior to the race and race day. Subjects were provided with a list of foods high in vitamins E and C to be avoided and they were instructed on how to read food labels to identify foods fortified with these nutrients.

All foods for the 2 days of the controlled diet were prepared and provided to the participants in the Metabolic Feeding Unit in the Human Nutrition Research Laboratory at Oregon State University. Subjects were then instructed to resume the restricted vitamin C and E diet for 6 days post race. Both diets consisted of 10-15% protein, 55-65% carbohydrate, and 25-30% fat; the macronutrient breakdown recommended for optimal athletic performance (45, 101). For the entirety of the study, subjects were provided with energy bars, carbohydrate pastes and carbohydrate drinks known to be low in vitamins E and C, to be used for fuel during their training and during the race.

Activity Log

For the six w prior to the ultramarathon, subjects recorded daily the amount, type, duration and intensity of exercise performed each day. This information was used to identify inter-subject differences in training regimen.

Non-Steroidal Anti-Inflammatory Drugs (NSAIDs)

Subjects were asked to refrain from using NSAIDs including naproxen sodium and ibuprofen throughout the seven w of the study. As an alternative to these drugs, subjects were allowed to use acetaminophen.

Race Schedule

Subjects were weighed immediately before and after the race to estimate losses in body water. Selected foods and beverages were provided to the subjects at check points along the race course, allowing for quantification of the caloric and nutrient intake of the subjects, and as a means to control vitamin E and vitamin C intake before and during the race. Subjects wore a Polar heart rate monitor (Polar Electro Inc., Woodbury, NY) during the run; average heart rate in conjunction with oxygen consumption levels, measured previously in the lab, were then used to quantify individual energy expenditure during the race. Subjects were asked to refrain from physical activity for the 6 days following the ultramarathon in order to investigate the effects of AO supplementation on recovery.

Energy Expenditure Calculation

Energy expenditure was calculated for each individual based on average heart rate during the run and the corresponding oxygen consumption (VO_2) multiplied by the time it took each subject to finish the race.

Average HR (bpm) - VO_2 (L/min) X 5 = kcal/min X Race Time (min)

=Total kcals

METHODOLOGIES

Assessment of Lipid Peroxidation

Plasma F_2-IsoPs were measured by stable isotope dilution gas chromatography negative ion chemical ionization mass spectrometry as described previously (166).

Plasma Antioxidants

Ascorbic and uric acids were determined by paired-ion reversed-phase HPLC coupled with electro-chemical detection using a modification of the method described by Kutnink et al. (153). α-Tocopherol was measured by HPLC coupled with electro-chemical detection using a modified version of the method of Podda et al. (152). Plasma triglycerides and cholesterol were measured by standard clinical assays (Sigma kit). Plasma α-tocopherol was standardized for changes in lipoproteins using plasma lipid levels (cholesterol + triglycerides).

Inflammatory Markers

Plasma cytokines and CRP were measured as reported previously; IL-1β and TNF-α (167), IL-6 and CRP (168).

Statistical Analyses

Data are expressed as the mean $\pm$ SE of 22 subjects. Analysis of covariance for repeated measures was used to detect statistically significant between and within subject effects (154). In order to adjust for pre-existing differences between individuals prior to supplementation, baseline concentrations of the following markers were used as covariates in the corresponding statistical model. Baseline covariates included: ascorbic acid ($p < 0.01$), α-tocopherol ($p < 0.003$), α-tocopherol/lipid ($p < 0.0001$), F_2-IsoPs (p

< 0.001), IL-6 (p < 0.002), TNF-α (p < 0.02) and CRP (p < 0.02). An unpaired t-test was used to analyze differences between genders with regard to subject characteristics (i.e. age, height, weight). Statistics were calculated using The SAS System (SAS Institute Inc, Cary, NC).

RESULTS

RACE RESULTS

Race results have been reported (14). Briefly, all 22 subjects completed the race (**Table 4**); previously only 21 were reported due to spurious findings for the comet assay in one subject (14). Run time averaged 423 $\pm$ 11 min at a pace of 13.7 $\pm$ 0.4 min/mile and an intensity of 71 $\pm$ 2 %VO$_2$ $_{max}$; there were no statistically significant differences in run time, pace or %VO$_{2max}$ between the genders or the treatment groups. Energy *expenditure* was approximately 2000 kcal greater for men than women and energy *intake* was greater for men than women (energy intake (kcal) 2530 $\pm$ 325 (AO) 2468 $\pm$ 279 (PL) for men compared with 1844 $\pm$ 137 (AO) 2040 $\pm$ 221 (PL) for women); neither parameter was different between AO and PL groups within each gender. Carbohydrate intakes were higher in men than women, but percent of total calories from carbohydrate did not differ between genders or treatment groups (**Table 4**). Intakes of vitamins E and C from food during the run were nominal, < 5 mg and < 50 mg respectively and did not differ between treatment groups or genders.

Table 4. Race Results.

Parameter	Females		Males	
	AO (N=6)	PL (N=5)	AO (N=6)	PL (N=5)
Run Time (min)	422 ± 17	436 ± 35	408 ± 22	430 ± 31
% VO_{2max}	74 ± 5	67 ± 3	71 ± 6	70 ± 7
CHO Intake (kcal) *	1626 ± 99	1636 ± 180	2030 ± 290	1895 ± 247
% CHO	78 ± 3	81 ± 4	80 ± 1	76 ± 3

Values are means $\pm$ SE. HR, heart rate; VO_{2max}, maximal oxygen consumption; CHO, carbohydrate.
* male vs females $p < 0.05$

PLASMA ANTIOXIDANTS IN RESPONSE TO SUPPLEMENTATION

In response to 6 w supplementation (14), plasma α-tocopherol increased in the AO group (28 ± 2 vs. 46 ± 3 μM, p < 0.05), but was unchanged in the PL group (24 ± 2 vs. 26 ± 2 μM). Similarly, ascorbic acid increased in the AO group (113 ± 14 to 127 ± 12 μM; p < 0.05) but was unchanged in the PL group (93 ± 11 vs. 73 ± 12 μM).

PLASMA ANTIOXIDANT RESPONSE TO THE RUN

Ascorbic acid concentrations were significantly higher in the AO group

Figure 10. Plasma ascorbic acid concentrations increased in response to supplementation and to the ultramarathon run. Ascorbic acid concentrations (mean ± SE) were significantly higher in the AO compared with the PL group at all time points except baseline (Treatment main effect, p < 0.0007). In response to the run, plasma ascorbic acid increased in both treatment groups (Time main effect, p < 0.002). Ascorbic acid concentrations were increased compared to pre-race at mid-race and post-race (p < 0.01), returning to pre-race values by 2 h post-race; there were no differences between men and women. Uncorrected baseline (Base) values used as the covariate in the model are also presented; Time 0 h = pre-race. (* = compared to pre-race).
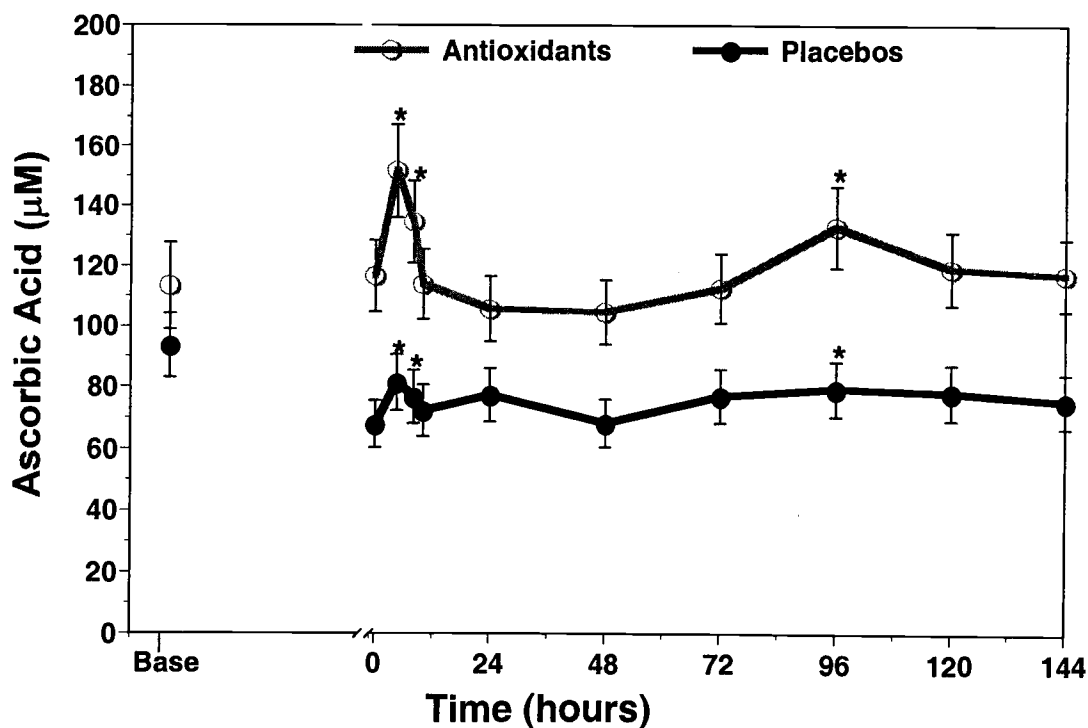
compared with the PL group at all time points except baseline (**Figure 10**, Treatment main effect, $p < 0.0007$). In response to the 50 km ultramarathon run, plasma ascorbic acid *increased* similarly in both treatment groups (Time main effect, $p < 0.002$) with significant increases compared to pre-race at mid-race and post-race ($p < 0.01$), returning to pre-race values by 2 h post-race. Men and women had similar ascorbic acid concentrations.

Concentrations of uric acid, a plasma water-soluble antioxidant (23), were not different between treatment groups, but were higher in men than in women at all time points (Gender main effect, $p < 0.01$). Adjustment for differences in pre-supplementation uric acid concentrations (baseline covariate, $p < 0.0002$) eliminated these gender differences; therefore, uncorrected uric acid concentrations are presented. Plasma uric acid concentrations increased in response to the run in both men and women (**Figure 11**, Time main effect, $p < 0.001$). Compared to pre-race, uric acid concentrations were elevated at mid-, post-, 2 h post- and 1 d post race ($p < 0.02$), not returning to pre-race until 2 d post-race.

Similar to ascorbic acid, α-tocopherol concentrations were significantly higher in the AO group compared with the PL group at all time points except baseline (**Figure 12A**, Treatment main effect, $p < 0.0001$). α-Tocopherol concentrations changed differently in the treatment groups over time (Treatment X Time interaction, $p < 0.007$). In the AO group compared to pre-race, α-tocopherol concentrations increased at mid-race ($p < 0.003$), returned to pre-race values by post-race, and fell to below pre-race concentrations for the duration of the study ($p < 0.01$). In the PL group, no changes in α-tocopherol concentrations were observed during the race, but declined to below pre-race values 2 and 5 days post-race.

Figure 11. Plasma uric acid concentrations increased in response to the ultramarathon run. Uric acid concentrations (mean ± SE) were significantly higher in men than in women at all time points (Gender main effect, p < 0.01), and increased in response to the run in both genders (Time main effect, p< 0.001). Compared with pre-race, plasma uric acid concentrations were elevated at mid-, post-, 2 h post- and 1 d post race (p < 0.02), not returning to pre-race until 2 d post-race. Uric acid concentrations were not different between treatment groups. Time 0 h = pre-race. Values shown are not corrected for differences between the genders at baseline (Base). (∗ = compared to pre-race).
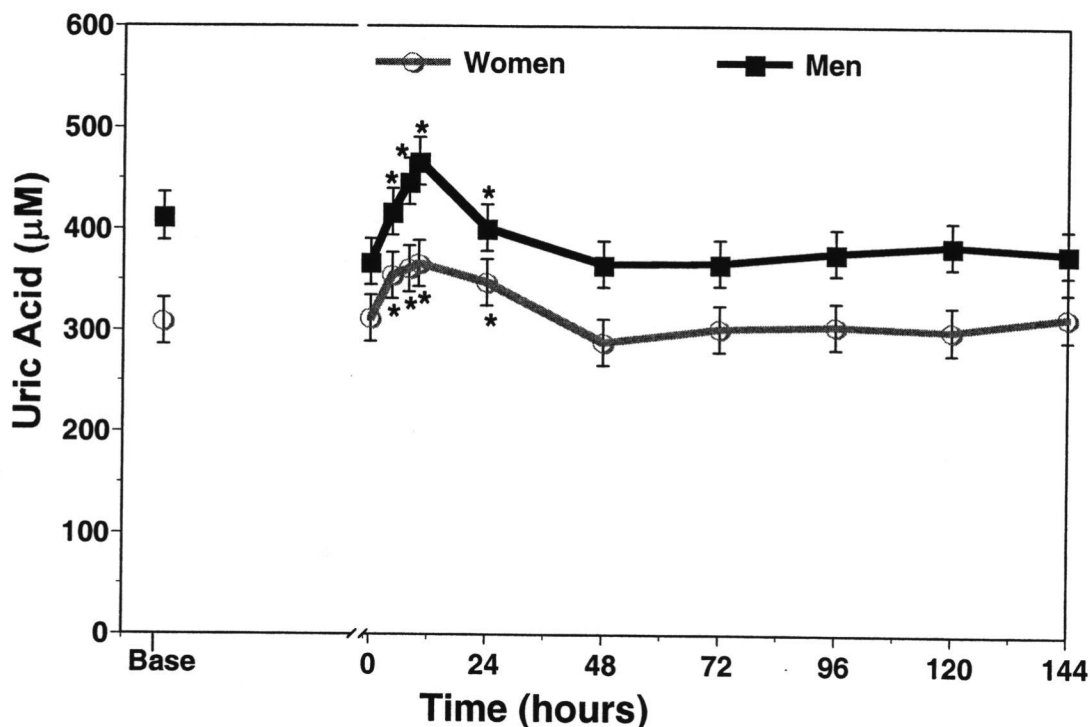
Lipid corrected α-tocopherol concentrations (per lipids = α-tocopherol / cholesterol + triglycerides) were also significantly higher in the AO group than the PL group at all time points except baseline (**Figure 12B**, Treatment main effect, p<0.0001). Compared to pre-race, α-tocopherol per lipids were decreased 3, 4, 5 and 6 days post-race in all subjects (**Figure 12B**, Time main effect, p< 0.003).

**Figure 12. Plasma α-tocopherol but not α-tocopherol/lipid
concentrations increased in response to the ultramarathon run.**
α-Tocopherol concentrations (mean ± SE) were significantly higher in the
AO compared with the PL group at all time points, except baseline
(Treatment main effect, p < 0.0001). α-Tocopherol concentrations changed
differently in the two treatment groups over time (Treatment X Time
interaction, p < 0.007). In the AO group, α-Tocopherol concentrations
increased at mid-race (compared with pre-race, p < 0.003), returned to pre-
race values by post-race, and decreased below pre-race concentrations for
the duration of the study (compared to pre-race, p < 0.01). α-Tocopherol
concentrations were unchanged in the PL group during the race, but
declined to below pre-race values 2 and 5 days post-race **(A)**.
α-Tocopherol/lipid ratios (mean ± SE) were significantly higher in the AO
group compared with the PL group at all time points except baseline
(Treatment main effect, p < 0.0001). α-Tocopherol/lipid concentrations
decreased similarly in both treatment groups at 3, 4, 5 and 6 days post race
(compared to pre-race, p < 0.01; Time main effect, p < 0.003) **(B)**.
Uncorrected baseline (Base) values used as the covariate in the model are
also presented; Time 0 h = pre-race. (* = compared to pre-race).
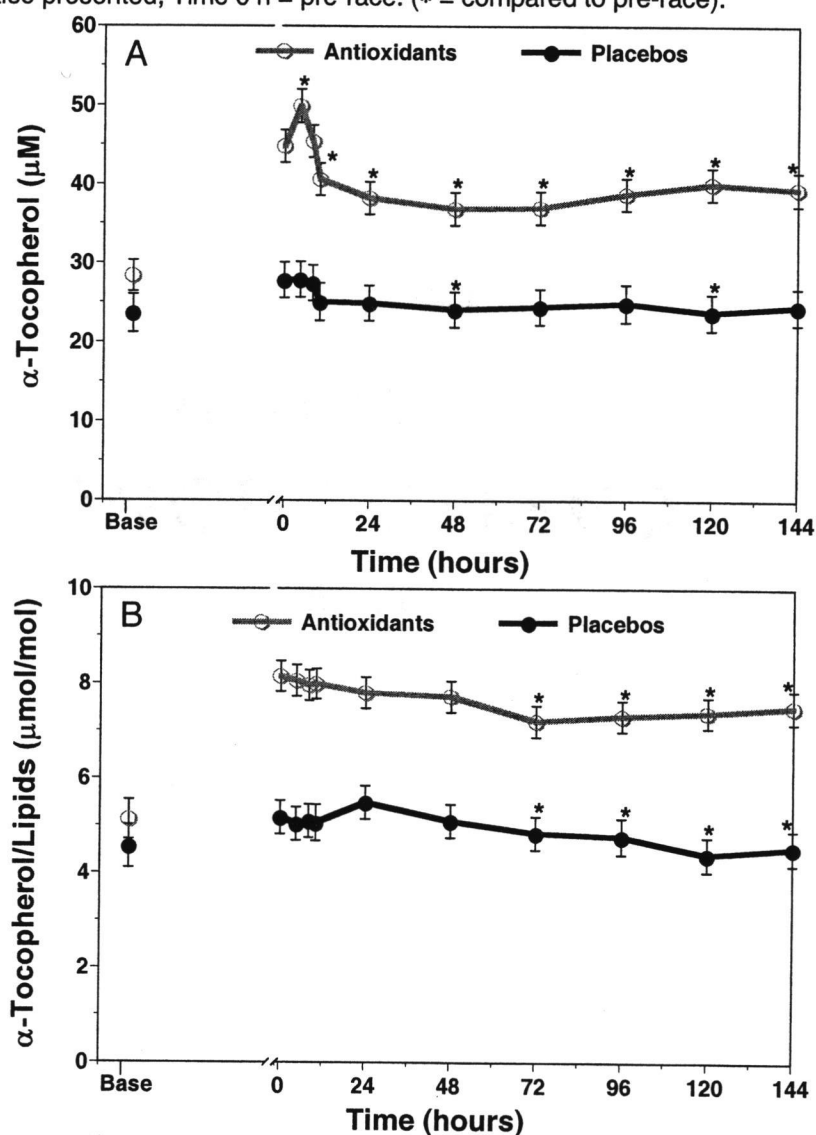
LIPID PEROXIDATION

At baseline (prior to supplementation), plasma F_2-IsoP concentrations were negatively correlated with VO_{2max} (R = -0.55, p < 0.01). There were no statistically significant differences detected between genders or treatment groups in F_2-IsoP concentrations at baseline, or following 3 and 6 w daily supplementation with AO or PL (**Figure 13**).

Figure 13. Antioxidant supplementation prevented increases in plasma F_2-IsoP concentrations following a 50 km ultramarathon. There were no statistically significant differences detected between genders or treatment groups in F_2-IsoP concentrations (mean ± SE) at baseline or following 3 and 6 w daily supplementation with vitamin E and vitamin C or placebos. At post-race F_2-IsoP concentrations were elevated in the PL group (compared to pre-race, p < 0.0001), but not in the AO group and were significantly higher in the PL group compared to the AO treatment group (AO vs. PL group, p < 0.001). No statistically significant differences were detected between genders prior to or during the race (ANOVA main effect for Time, p < 0.0001; main effect for Treatment, p < 0.04; and significant Treatment X Time interaction, p < 0.01). (* = compared to pre-race; # = AO vs. PL group).
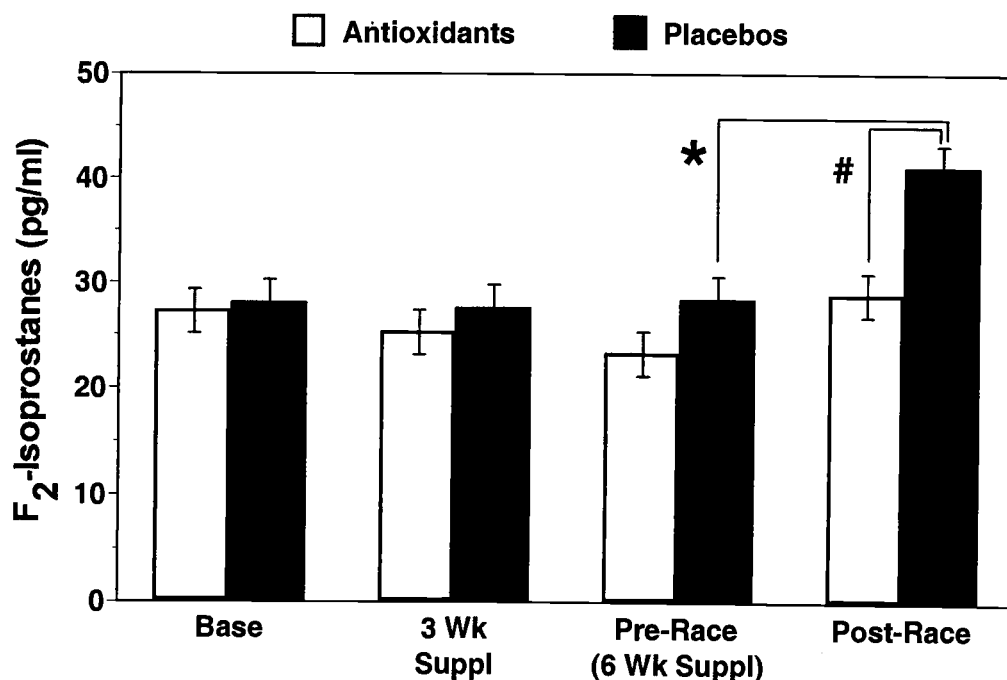
F_2-IsoP concentrations increased significantly at post- compared with pre-race *only* in the PL group (28 ± 2 to 41 ± 3 pg/ml; p < 0.0001; **Figure 13**). Moreover, at post-race F_2-IsoP concentrations in the PL group were significantly higher than in the AO group (p < 0.001). There were no statistically significant differences detected between genders during the run (ANOVA, main effect for Time, p < 0.0001; main effect for Treatment, p < 0.04; and Treatment X Time interaction, p < 0.01).

While there were no differences in F_2-IsoP concentrations between genders prior to or during the race, men and women did respond differently to the treatments *following* the ultramarathon (Gender X Treatment interaction, p < 0.03). In women, F_2-IsoP concentrations were elevated in the PL compared with the AO group at post-race (p < 0.01), but they returned to pre-race concentrations by 2 h post-race and did not differ from the AO women for the duration of the study (**Figure 14A**). By contrast, F_2-IsoP concentrations in men were higher in those taking PL than in those taking AO at post-, 2 h post and for 1, 2, 3, 4, and 6 days post race (PL vs. AO, p < 0.03; **Figure 14B**).

Using paired data from each subject at every time point, plasma F_2-IsoP concentrations were negatively correlated with α-tocopherol/lipids; R = -0.40, p < 0.0001). At post-race, when F_2-IsoP concentrations were maximal, concentrations were negatively correlated both with α-tocopherol/lipids (R = -0.61, p < 0.003) and ascorbic acid (R = -0.41, p = 0.05).

Figure 14. Plasma F_2-IsoP concentrations in women and men 1 h prior to, during and 6 days after competition in a 50 km ultramarathon. F_2-IsoP concentrations (mean ± SE). Men and women responded differently to the treatment in the hours and days following the ultramarathon (Gender X Treatment interaction, p < 0.03). **(A)** In women, F_2-IsoP concentrations were elevated in the PL group compared with the AO group at post-race, but not at later time points (AO vs. PL group, p < 0.01). **(B)** In men, F_2-IsoP concentrations were higher in the PL group compared with the AO group at post-, 2h post- and 1, 2, 3, 4, and 6 days post-race (AO vs. PL group, p < 0.03). Uncorrected baseline (Base) values used as the covariate in the model are also presented; Time 0 h = pre-race. (* = compared to pre-race; # = AO vs. PL group).
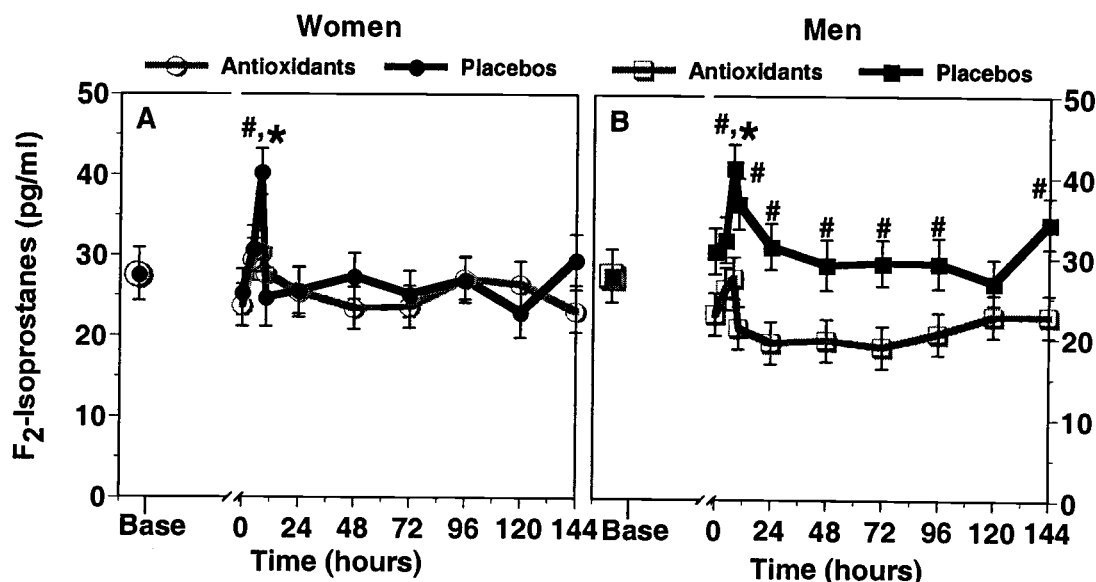

INFLAMMATORY MARKERS

Inflammation may be mediated by ROS (25, 106), therefore, we also measured markers of inflammation. The ultramarathon run elicited dramatic increases in most of these markers, but AO supplementation did not appear to have an effect on any of the selected parameters. For example, IL-6 concentrations increased during the race similarly in both AO and PL groups (**Figure 15**, Time main effect, p< 0.0001); but by 1-day post-race, plasma IL-6 concentrations returned to pre-race values. Interestingly, F_2-IsoP

concentrations were correlated with IL-6 at mid-race (R = 0.46, p < 0.03;

Figure 16), but not at other time points.

Figure 15. Plasma IL-6 1-hour prior to, during and immediately following a 50 km ultramarathon. IL-6 concentrations (mean ± SE) increased in response to the ultramarathon (Time main effect, p < 0.0001) and were elevated at mid-, post- and 2 h post-race (compared to pre-race, p < 0.0001). IL-6 concentrations were highest at post-race (compared to mid-race, p < 0.004). Uncorrected baseline (Base) values used as the covariate in the model are also presented; Time 0 h = pre-race.
(* = compared to pre-race; & = compared to mid-race).
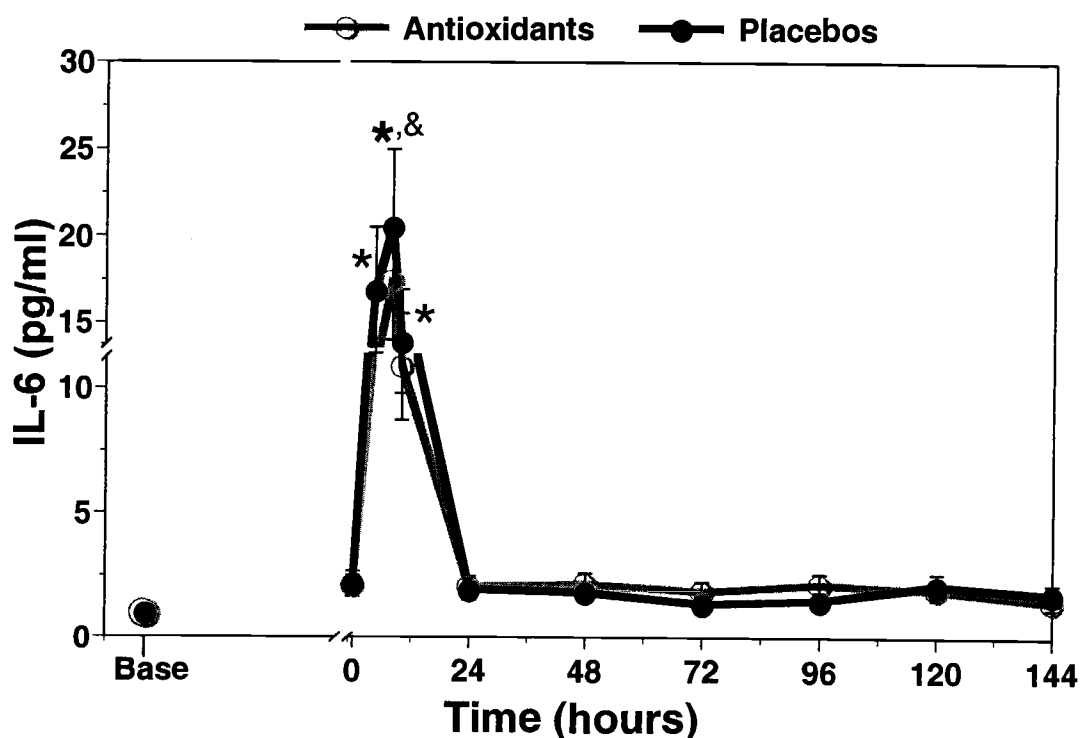
The time course of inflammatory markers up to 48 h post-race is

depicted in **Figure 17**. TNF-α behaved similarly to IL-6, increasing as early as

mid-race in response to the ultramarathon (Time main effect, p< 0.0001).

CRP increased following the run (**Figure 17**, Time main effect, p< 0.0001). At

2 d post-race, CRP plasma concentrations had declined to 8.2 ± 0.9 mg/dL (p

< 0.0001) but at study end (6 d post-race), plasma CRP concentrations

remained twice as high as pre-race concentrations (p < 0.0001; data not

shown). IL1-β did not change significantly in response to the exercise

bout, nor did it appear to be affected by antioxidant supplementation (data not

shown).

Figure 16. At mid-race, plasma IL-6 was significantly correlated with F_2-IsoP concentrations (R = 0.46, p < 0.03).
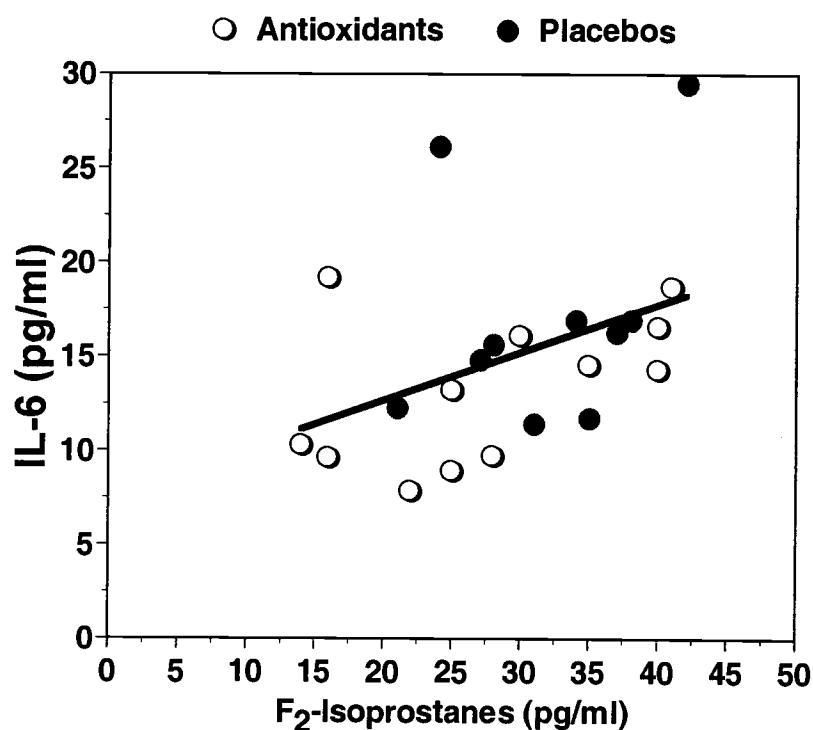
Figure 17. Time course of inflammatory markers 1 hour prior to, during and up to 48 hours post-race. Inflammatory marker concentrations (mean ± SE). Plasma IL-6 and TNF-α concentrations were both elevated at mid-race, remained elevated at post- and 2 h post-race. C-Reactive protein (CRP) was not elevated until post-race, and did not achieve maximum values until 1 d post-race, remaining elevated 48 h post race (compared to pre-race, p < 0.0001). Uncorrected baseline (Base) values used as the covariate in the model are also presented; Time 0 h = pre-race. (* = compared to pre-race).
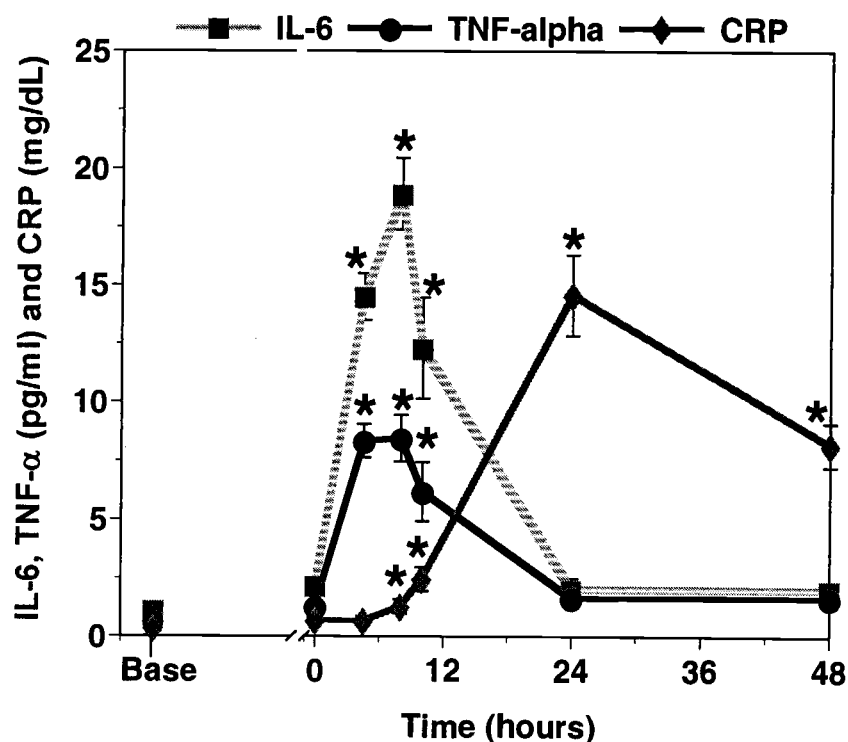
DISCUSSION

Supplementation with vitamins E and C completely inhibited exercise-induced lipid peroxidation (**Figures 13 and 14**). At post-race, when oxidative stress was maximal, F_2-IsoP concentrations were highly negatively correlated both with α-tocopherol/lipids (R= -0.61, p<0.003) and ascorbic acid (R= -0.41, p= 0.05) offering further evidence that antioxidants were responsible for preventing lipid peroxidation. These findings are in contrast to the lack of effects of antioxidants on exercise-induced DNA damage that we reported previously in these subjects (14).

Surprisingly, men and women responded differently to AO supplements in the days following the ultramarathon. PL women recovered rapidly following the race with no differences in F_2-IsoP concentrations between the two treatment groups during the week following the ultramarathon. In contrast, elevated F_2-IsoP concentrations persisted in PL men during the recovery phase. It should be noted that subjects were instructed to refrain from physical activity for the 6 days following the run.

Plasma F_2-IsoP concentrations at baseline were negatively correlated with VO_{2max} (R = -0.55, p < 0.01) suggesting that cardiovascular fitness was inversely related to lipid peroxidation as has been previously proposed (169). Subjects were recreationally trained with an average VO_{2max} of, 58 ± 1 ml/kg/min, making results of the present study generalizable to the broader physically active population but not necessarily to sedentary persons or elite athletes. Subjects were randomly allocated to treatment groups, therefore VO_{2max} and F_2-IsoP concentrations were initially similar between groups and unlikely to be confounding variables.

F_2-IsoPs are specific end products of cyclooxygenase-independent, free-radical catalyzed oxidation of arachidonic acid (76) and are a more sensitive and reliable measure of in vivo lipid peroxidation than previous assays such as malondialdehyde (MDA) and lipid hydroperoxides (76). Muscle damage caused by the resumption of weight bearing activity following space flight increased urinary excretion of F_2-IsoPs, implicating oxidative stress as a cause of muscle damage (120). In the present study, F_2-IsoPs increased only after subjects experienced an exceptional oxidative insult, the ultramarathon, and then only in the PL group (**Figure 13**). We demonstrated that this oxidative stress was sufficient to cause DNA damage in both AO and PL groups (14). Most (81-83), but not all (84), studies of high doses of vitamin E on steady state levels of F_2-IsoPs in healthy, non-smoking adults have concluded no effect of the antioxidant. Combinations of antioxidants have provided more positive findings. Upritchard et al. (85) reported that 111 mg/day vitamin E and 1.24 mg/day carotenoids for 11 w reduced plasma F_2-IsoPs by 15 %. Supplementation with vitamins E and/or C (~500 mg/d and/or 400IU/day respectively) for a longer duration (8 w) attenuated urinary excretion of F_2-IsoPs (86).

With regard to exercise-induced lipid peroxidation, few exercise studies have utilized F_2-IsoPs as a biomarker of lipid peroxidation. Mori et al. (121) trained non-insulin dependent diabetics 30 minutes per day at 55-65% VO_{2max} on a cycle ergometer 3 times per w for 8 w. They reported no increase in urinary F_2-IsoPs in response to the moderate exercise training protocol suggesting that the exercise bout did not elicit an oxidative stress response. Sacheck et al. (32) reported in young and elderly men effects of supplementation with 1000 IU RRR-α-tocopherol for 12 w prior to a 45 minute

downhill run. Vitamin E attenuated the increase in plasma F_2-IsoPs in the elderly men at 24 h post-exercise, but not at 72 h post exercise. In the young men, supplementation prevented any increases in plasma F_2-IsoPs, but the group started out with higher levels at baseline than the placebo group making interpretation of the results difficult. Since F_2-IsoPs were not measured immediately post-exercise, comparison with the present study, where F_2-IsoPs peaked at post-race, is not possible.

In the present study, despite similar lipid peroxidation responses during the run, men and women responded differently in the days following the race. F_2-IsoP concentrations in PL women recovered rapidly whereas higher levels persisted in PL men for the duration of the study despite similar plasma antioxidant levels in the two PL groups. Greater oxidative stress in men than in women has been reportedly previously. Ide et al. (87) studying healthy non-smoking adults, reported 2-fold higher excretion of urinary of F_2-IsoPs in men than in women. However, following 4 w supplementation with vitamins E and C, F_2-IsoP concentrations in men were reduced to those observed in the women (87). One explanation for the observed gender difference is a higher metabolic rate in men leading to increased mitochondrial flux and increased production of ROS (87). Women and men in the present study were of similar fitness levels, but as is typical, men exhibited a higher proportion of lean body tissue (14), likely contributing to a higher metabolic rate (101). Another possible explanatory variable, the female hormone estrogen, is known to exhibit antioxidant properties (87) and thus may have contributed to the faster recovery observed in PL women.

Very few studies have examined the effects of antioxidants on both exercise-induced oxidative stress and inflammation (37, 41). Nieman et al.

(37) reported no effect of 1500 mg/day ascorbic acid for one w prior to an ultramarathon on exercise-induced increases in plasma F_2-IsoPs, lipid hydroperoxides or IL-6. Using an eccentric arm exercise protocol known to induce muscle damage, Childs et al. (41) studied the effects of 1 w *post-exercise* supplementation with ascorbic acid and N-acetyl-cysteine (NAC). Both F_2-IsoPs and lipid hydroperoxides increased in response to the exercise, but levels were higher in the supplemented group post-exercise. Childs et al. (41) suggested a pro-oxidant effect of ascorbic acid and NAC. Nonetheless, these supplements had no effect on exercise–induced increases in IL-6. In the present study, IL-6 increases in response to the ultramarathon were not modulated by vitamin E and C supplementation. Similarly, in the present study, CRP was not attenuated by AO supplementation, in accordance with previous reports (170).

The only inflammatory marker not changed by the ultramarathon run was IL1-β; it too was unaffected by AO supplementation. While previous studies of *untrained* subjects have reported increases in IL1-β with exercise (104, 106) studies have consistently reported no response in *trained* exercisers (118) except a slight but significant increase observed in ultra-endurance runners (90 km ultramarathon) (116).

As carbohydrate ingestion is known to modulate the cytokine response to exercise (171), carbohydrate intake was monitored and recorded prior to, during and for 2 hours following the race. Carbohydrate intake was higher in men than in women, but was not different between treatment groups within each gender (**Table 4**). Carbohydrate intake as a proportion of total calories was similar between genders and treatment groups and therefore unlikely to have influenced study outcomes with regard to cytokines.

Ascorbic acid is the most effective antioxidant in human plasma (28); it can prevent initiation of lipid peroxidation and spare other critical antioxidants including α-tocopherol and urate (28). In response to the 50 km ultramarathon run, plasma ascorbic acid *increased* similarly in both treatment groups with significant increases compared to pre-race, at mid-race and post-race returning to pre-race values by 2 h post-race. Similar increases in plasma ascorbic acid in response to vigorous exercise have been reported previously (1, 7, 13, 48, 107, 110), although some studies have reported no change (31, 111, 112). It has been suggested that exercise-related increases in cortisol secretion promote efflux of ascorbic acid from the adrenal gland (115) and/or the mobilization of ascorbic acid from other tissue sites such as leukocytes or erythrocytes (48). Some (111, 112, 116), but not all (37), recent studies have demonstrated an attenuation of the exercise related increase in circulating cortisol with vitamin C supplementation.

Uric acid, a primary end product of purine metabolism and a water-soluble antioxidant (23) increased in the present study, consistent with the findings of others (13, 49, 100). The increase may be explained by enhanced purine oxidation with exercise (13, 49, 100). Increased energy requirements characteristic of vigorous exercise upregulate various metabolic pathways including adenylate cyclase (43, 49) (or myokinase in muscle (101)). This enzyme is responsible for production of 1 ATP and 1 AMP from 2 ADP (101)). While the ATP is used for energy, the AMP is degraded to uric acid (102). Concurrent increases in plasma ascorbic and uric acids may reflect enhanced antioxidant defenses in response to the oxidative stress of the endurance exercise. Moreover, our findings are in accordance with previous studies

reporting increased antioxidant enzymes (11) and antioxidant nutrients (6, 13, 48) in response to extreme exercise.

We observed an increase in plasma α-tocopherol concentrations during the exercise in the AO, but not the PL group. After correcting α-tocopherol for lipids, no significant changes in α-tocopherol/lipids were observed in either group. Thus, the increase in plasma α-tocopherol concentrations during the exercise could be completely explained by fluctuations in lipids during the run. A few of the studies investigating the effects of vitamins E and/or C supplementation on endurance running have reported increases in plasma α-tocopherol concentrations in both supplemented and placebo groups following exercise (13, 40, 103) but most failed to report post-exercise plasma α-tocopherol concentrations (31, 32, 104-106). A single study reported no change in α-tocopherol concentrations with exercise (107). Only one of these studies reported both absolute α-tocopherol concentrations and α-tocopherol/lipid concentrations (103) and observed differential responses in the AO supplemented groups that depended on correction for fluctuations in lipid levels.

As presented schematically in **Figure 18**, ROS generated in response to exercise can cause oxidative damage (1) and stimulate an inflammatory response (106). Therefore it has been proposed that AO supplementation could prevent exercise–induced oxidative stress and protect against both oxidative damage and inflammation (37, 106). Unlike previous studies testing this hypothesis, the present study investigated the effects of supplementation with *both* vitamins E and C, measuring oxidative stress *and* inflammatory markers in response to exercise. Supplementation *prevented* oxidative damage (lipid peroxidation), but had no apparent effect on inflammation.
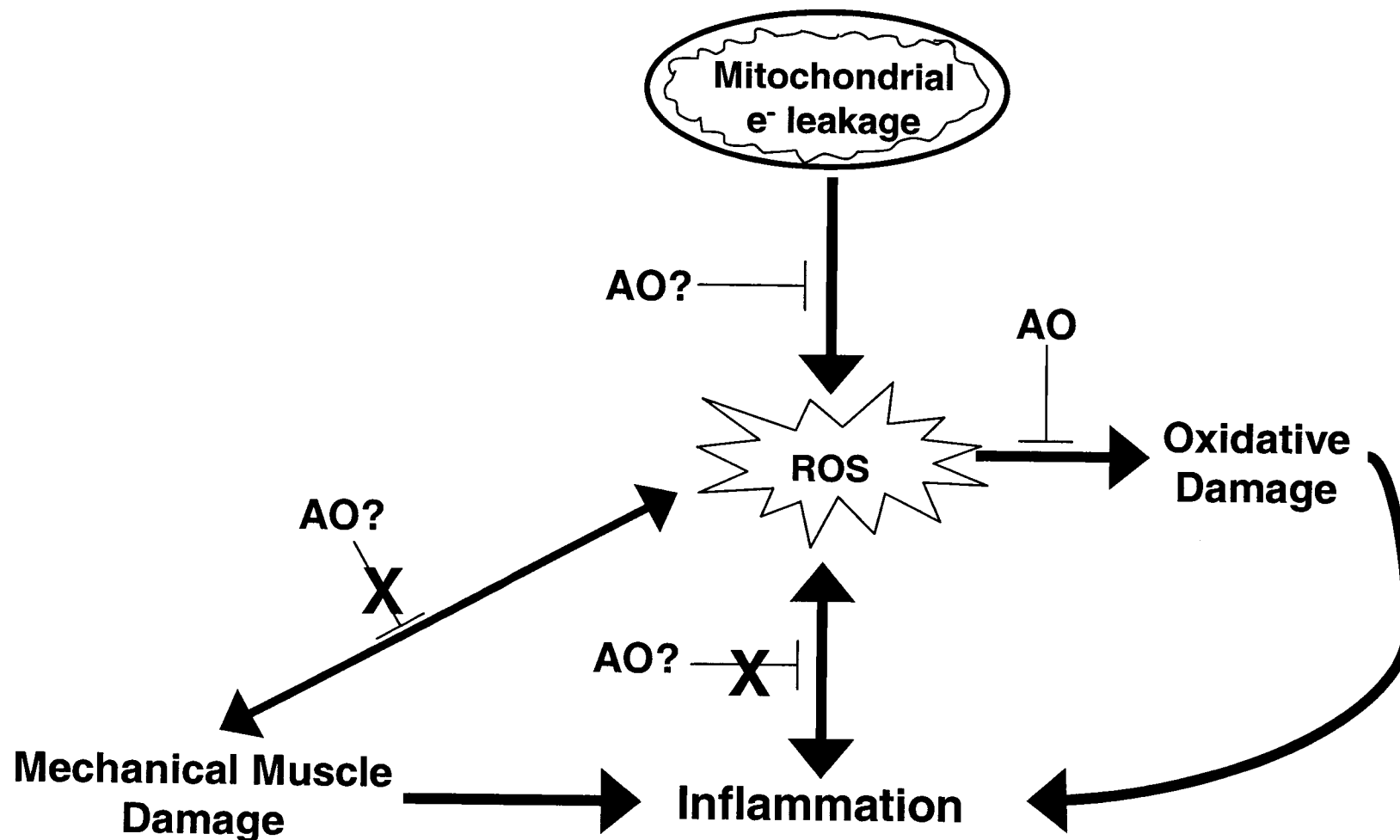
Figure 18. Proposed Model of Exercise Induced Oxidative Stress and Inflammation. With exercise there is increased flux of oxygen through the mitochondria, electron (e-) leakage, and generation of reactive oxygen species (**ROS**) that may exceed antioxidant defenses. These excess ROS can: **1)** cause oxidative damage and **2)** stimulate an inflammatory response. Exercise-induced mechanical muscle damage may or may not lead to a delayed inflammatory response (31). Hypothetically, supplementation with antioxidants (**AO**) prevents (⊥) exercise–induced oxidative stress and may therefore protect against both oxidative damage and subsequent inflammation. We demonstrate that supplementation with vitamins E and C *prevents (⊥) oxidative damage* (lipid peroxidation), but has no apparent effect on inflammation (**X**). These results suggest that oxidative damage and the inflammatory response are operating independently. It may be that ROS do not modulate inflammation in this model or that the inflammation induced by muscle damage is great enough to overwhelm the protective effects of the antioxidants.

These results suggest that oxidative damage and the inflammatory response are operating independently (37). Alternatively, it may be that higher doses of α-tocopherol ($\geq$ 800 IU/day) are required to elicit an anti-inflammatory effect, as has been previously suggested (168, 172).

Preventing production/enhancing clearance of F_2-IsoPs may be more biological relevant than preventing inflammation. F_2-IsoPs have demonstrated pro-atherogenic biological activity, including vasoconstriction, and activation of platelet aggregation (37, 76) and they are known to recruit pro-atherogenic monocytes and induce monocyte adhesion (173). In contrast, the exercise-induced inflammatory response stimulates recovery from exercise by inducing regeneration of damaged tissue and recruitment of satellite cell proliferation (146). Together, AO supplementation proved to prevent the damaging increase in lipid peroxidation without influencing inflammation. This is especially important since prevention of exercise-induced inflammation could actually inhibit muscular adaptation to physical activity, the so-called "training effect" of exercise.

The acute oxidative and inflammatory stress responses characteristic of vigorous aerobic exercise are analogous to the stress responses observed following acute events such as myocardial infarction, ischemic stroke, surgery and trauma (25, 145, 174). Thus, aerobic exercise provides a well-controlled, relatively non-invasive model to study the effects of AO supplementation on acute oxidative and inflammatory stress. The results from such research may be useful for the exploration of these other chronic human diseases.

CHAPTER 4: VITAMIN E KINETICS DEMONSTRATE ACCELERATED VITAMIN E DISAPPEARANCE IN WOMEN COMPARED TO MEN IN RESPONSE TO AN ULTRAMARATHON RUN

Angela Mastaloudis[1,2], Scott W. Leonard[2], Sekhar Ramakrishnan[3] and Maret G. Traber[1,2,4]

[1]Department of Exercise and Sport Science, Oregon State University, Corvallis, OR; [2]Linus Pauling Institute, Corvallis, OR; [3]College of Physicians and Surgeons, Columbia University, New York, NY; [4]Department of Nutrition and Food Management, Oregon State University, Corvallis, OR

Vitamin E Kinetics Demonstrate Accelerated Vitamin E Disappearance in Women Compared to Men in Response to an Ultramarathon Run

ABSTRACT

Vigorous exercise induces lipid peroxidation (LO) and accelerated plasma α-tocopherol (α-TOH) disappearance. To determine if 6 w supplementation with vitamins E and C could moderate α-TOH turnover by attenuating exercise-induced LO, 22 runners were studied during a 50 km run, following random assignment to treatment: 1) placebos (PL) or 2) antioxidants (AO;1000 mg vitamin C and 400 IU RRR-α-tocopheryl acetate). 48 h prior to the race subjects consumed 75 mg deuterium labeled vitamin E (d_6-RRR α-tocopheryl acetate (d_6-α-TOH)). Blood samples were obtained before supplementation (baseline), 24 h pre-, 12 h pre-, and 1 h pre-race, mid-race, post-race, 2 h post-race and for 6 d post-race. Plasma labeled and unlabeled α-TOH, vitamin E metabolites (α- and γ-CEHCs), triglycerides, cholesterol and ascorbic acid were measured. Plasma d_6-α-TOH concentrations were similar in the AO and the PL groups 24 h after consumption of d_6-α-TOH. Plasma d_6-α-TOH exponential disappearance rates were calculated for: Pre-race (24-48 h following d_6-α-TOH administration, (Rate 1)), Race (Rate 2), and Recovery (Rate 3). Disappearance was faster during the Race than Pre-Race. Rates were faster in the AO than in the PL group, and faster in women compared to men (regardless of treatment group), for all 3 periods. When plasma d_6-α-TOH was corrected for lipid concentrations, disappearance rates were similar during Race and Pre-race, suggesting that lipid fluxes account for faster d_6-α-TOH disappearance during the run. Increased disappearance of d_6-α-TOH in women compared to men and in AO supplemented versus PL are novel findings meriting further research.

INTRODUCTION

Vigorous exercise results in oxidative stress (21) as evidenced by increased lipid peroxidation (1), DNA damage (14) and protein oxidation (53). In response to endurance exercise, oxygen (O_2) consumption increases 10 to 20-fold systemically and as much as 100 to 200-fold at the level of the skeletal muscle resulting in substantially increased mitochondrial electron flux (6). Reactive oxygen species (ROS) leaking from the mitochondria during exercise are considered a main source of oxidative stress (23). Other potential sources of sources of ROS during exercise include enhanced purine oxidation, damage to iron-containing proteins, disruption of Ca^{2+} homeostasis (24), neutrophil activation (8) and autoxidation of catecholamines (3).

Previously, we demonstrated that endurance exercise stimulates a faster depletion of plasma deuterium-labeled α-tocopherol in addition to an increase in lipid peroxidation (1). We studied recreationally trained runners during a 50 km ultramarathon (trial 1) and a sedentary day (trial 2) 1 month later. Subjects consumed a controlled diet for 5 days during each trial. The night before the race (or sedentary trial), subjects consumed 75 mg each d_3-*RRR*- and d_6-*all rac*-α-tocopheryl acetates. Deuterated α-tocopherol disappearance rates were faster ($2.8 \times 10^{-4} \pm 5.4 \times 10^{-5}$) during the race compared to the sedentary trial ($2.3 \times 10^{-4} \pm 6.4 \times 10^{-5}$; $p < 0.03$) (1). Moreover, F_2-IsoPs nearly doubled during the race (pre-race vs post-race, 76 ± 24 vs 130 ± 54 pg/ml, $p < 0.0001$), but were unchanged during the rest trial (1). The increased rate of vitamin E disappearance in conjunction with increased F_2-IsoPs suggests that the runners experienced oxidative stress in excess of antioxidant protection.

Given that α-tocopherol disappearance is faster during exercise, when lipid peroxidation is maximal, the obvious conclusion is that lipid peroxidation increased vitamin E oxidation. However, since vitamin E is transported in lipoproteins, increased lipoprotein turnover could also be an explanation for vitamin E disappearance. The purpose of this study was to evaluate whether decreasing oxidative stress by increasing antioxidant protection by prior supplementation with the antioxidants (vitamins E and C) could moderate the rate of disappearance of deuterium labeled α-tocopherol.

EXPERIMENTAL PROCEDURES

HUMAN SUBJECTS

A description of the study design has been published previously (2, 14). Briefly, the protocol for this study was approved by the Oregon State University Institutional Review Board for the Protection of Human Subjects. Runners (11 women and 11 men) were recruited from the pool of participants in a 50 km (32 mile) ultramarathon trail run.

The physical characteristics of the subjects have been reported previously (2, 14). Average age was 39 ± 2.5 yrs, VO_{2max} 58 ± 1 ml/kg/min and estimated weekly training distance 43 ± 3 km; there were no differences between treatment groups or genders for these characteristics. Men were taller, weighed more and had lower percent body fat than women. Women in the AO group weighed slightly less than women in the PL group ($p < 0.05$) while men in the AO group were slightly taller than men in the PL group ($p < 0.05$). Within each gender, VO_{2max} and % body fat did not differ among treatment groups. Estimated average daily nutrient intakes: 2653 ± 201 kcal,

142 ± 26 mg vitamin C and 14 ± 3 mg vitamin E; there were no differences between men and women in energy, dietary vitamin E or C intakes.

CRITERIA FOR SUBJECT PARTICIPATION

Inclusion criteria for participation in the study included non-smoking status, age 18-60 y, and a VO_{2max} classified as excellent fitness by Powers and Howley (45). Potential participants were excluded based on antioxidant supplement use (e.g. vitamin C, vitamin E, selenium or carotenoids), abnormal cholesterol (≥ 7.8 mmol/L (300 mg/dl)), triglyceride (≥ 3.8 mmol/L (300 mg/dl)) or fasting blood glucose levels (≥ 7.8 mmol/L (140 mg/dl)) or other supplement use (performance enhancing, or herbal type products), vegetarian or other restrictive dietary requirements, and pregnancy or suspected pregnancy.

STUDY DESIGN

Randomization to Treatment Group

Subjects were randomly assigned in a double-blind fashion to one of two treatment groups 1) PL (300 mg soybean oil and 1000 mg citric acid (500 mg twice daily) or 2) AO (300 mg *RRR*-α-tocopheryl acetate [2,5,7,8-tetramethyl-2*R*-(4'*R*,8'*R*,12-trimethyltridecyl)-6-chromanol] in soybean oil and 1000 mg ascorbic acid (500 mg twice daily)).

Labeled Vitamin E Administration

The labeled vitamin E (*RRR*-α-5,7-$(CD_3)_2$-tocopheryl acetate) was provided by the Cognis Corporation, who attests to its purity for consumption by humans. The subjects in both treatment groups consumed a single gelatin capsule containing deuterium labeled vitamin E (75 mg d_6-*RRR* α-tocopheryl acetate) 48 h prior to the race (time 0) with a standardized breakfast containing 35% fat.

Blood Samples

Blood samples were obtained prior to antioxidant or placebo supplementation (baseline), the day before the race (24 h pre-race), the evening before the race (12 h pre-race), 1 h prior to the race (pre-race, 0h), in the middle of the race at kilometer 27 (mid-race, 5 h), immediately post-race, 2 h after race end (2 h post-race, 10 h) and for six days after the race (post 1-6 days, 24-144 h), for a total of 13 time points. All were fasting morning blood draws except 12 h pre-, mid-, post- and 2h post-race.

ANALYTICAL METHODS

Materials

Standards including unlabeled (d_0) and d_6-*RRR*-α-tocopheryl acetates were gifts from Dr. James Clark of Cognis Nutrition and Health, LaGrange, IL. *all rac*-α-5,7,8-$(CD_3)_3$ tocopheryl acetate was provided by Dr. Carolyn Good of General Mills and was synthesized by Isotec Inc. (Miamisburg, OH). The isotopic purity was found to be 88.4% d_9 and the remainder d_8. α-CEHC was a gift from W.J. Wechter. γ-CEHC was obtained from Cayman Chemicals (Ann Arbor, MI). Non-labeled γ-tocopherol, ascorbic acid, potassium hydroxide (KOH), butylhydroxy toluene (BHT), lithium perchlorate, trolox and β-glucuronidase (type H-1, contains minimum 300,000 U/g b-glucuronidase activity and minimum 10,000 U/g sulfatase activity) from Sigma-Aldrich (St. Louis, MO). Diethyl ether was obtained from Mallinckrodt Baker, Inc. (Phillipsburg, NJ), and HPLC-grade methanol, hexane, ethanol and glacial acetic acid were obtained from Fisher (Fair Lawn, NJ).

Extraction of Plasma Labeled and Unlabeled Tocopherols

Plasma vitamin E was extracted using the method of Podda et al. (152). In brief, a known amount of d_9-α-tocopherol (as the internal standard) was added to the sample, which was then saponified with alcoholic KOH, extracted with hexane, and the tocopherol contents analyzed by LC-MS (see below). Calibration curves were prepared using d_6- and d_9-α-tocopherols, as well as authentic unlabeled α- and γ-tocopherols.

Extraction of Plasma CEHCs

Plasma CEHCs were extracted using a modified method of Lodge et al. (175). In brief, a known amount of trolox (as the internal standard) was added to 0.5 mL plasma, before incubation for 30 min at 37°C with 100 µL β-glucuronidase (10 mg/mL 0.01 M potassium phosphate buffer, pH 6.8), and 10 µL of H_2O containing 1% ascorbic acid. After incubation, the samples were acidified by the addition of 10 µL 12 M HCL. CEHCs were extracted with 5 mL of diethyl ether. An aliquot of the ether fraction was dried under N_2 and the residue resuspended in 1:1 v:v H_2O:MeOH, containing 0.05% v:v acetic acid and 0.05% w:v ascorbic acid.

Liquid Chromatography

For LC-MS analysis a Waters (Milford, MA) 2690 Separations Module with a cooled auto sampler and a degassing unit was used. Instrument control and data acquisition were performed using Waters Masslynx V. 3.4 software.

Labeled and Unlabeled Tocopherols

The column used was a Supelcosil™ LC-18 column (7.5 cm x 4.6 mm, 3 µm particle, Supelco, Bellfonte, PA) with a Supelguard™ LC-18 precolumn (2 cm x 4.0 mm, 5 µm particle, Supelco, Bellfonte, PA). An isocratic mobile

phase (100% methanol) was delivered at 1 ml/min, with a total run time of 4 minutes.

α- and γ-CEHCs

The column used was a SymmetryShield™ RP-18 column (3.0 cm x 150 mm, 3.5 μm particle, Waters, Milford, MA) with a SymmetryShield™ Sentry™ RP-18 precolumn (3.9 cm x 20 mm, 3.5 μM particle, Waters, Milford, MA). For separation, a modified gradient method of Himmelfarb et al. (176) was used. The system was first equilibrated with 50:50 H_2O:MeOH for 1 min, followed by a linear gradient to 80% methanol (containing 0.05% acetic acid) at 6 min at a flow rate of 0.25 mL/min. These conditions were maintained for 15 min, followed by a 5 min wash period with 95% MeOH (containing 0.05% acetic acid), at which time original conditions were returned to and run for 5 min prior to injection of the next sample.

Mass-spectrometry

Labeled and Unlabeled Tocopherols

For mass spectral analysis, a ZQ 2000 single-quadrupole mass spectrometer (MS, Micromass, Manchester, England) was used, with the Micromass MassLynx NT version 3.4 software, using a modification of the described method (177). The source was equipped with an atmospheric pressure chemical ionization (APCI) probe, set to negative ionization mode. The analysis parameters were set as follows: corona discharge electrode 15.0 μA, APCI probe temperature 450° C and heater gas (nitrogen) 350 L/h, nebulizer gas (nitrogen) 80 psi, cone gas (nitrogen) 25 L/h and voltage -35 V; with a dwell time of 0.20 s per compound. All samples were analyzed using single ion recording (SIR). Mass-to-charge (*m/z*) ratios were obtained as

follows: d_0-α-tocopherol, m/z 429.4, d_9-α–tocopherol, m/z 438.4, d_6-α–tocopherol, m/z 435.4, and d_0-γ-tocopherol, m/z 415.4.

α- and γ-CEHCs

Samples were analyzed using the ZQ 2000 MS with an electrospray ionization source (ESI). The capillary was set to 2.5 kV, cone voltage -30 V, desolvation temperature at 150°C. The desolvation gas (nitrogen) was set to 160 L/hour, the nebulizer gas (nitrogen) at 80 psi, and the cone gas (nitrogen) at 50 L/h. SIR m/z ratios were obtained for d_0-α-CEHC, m/z 277.8; d_6-α-CEHC, m/z 283.8; d_0-γ-CEHC, m/z 263.8; d_2-γ-CEHC, m/z 265.8 and trolox, m/z 249.8. The dwell time for each of the ions was set at 0.20 s. Typical HPLC retention times were 14.2, 14.6, and 15.4 min for trolox, γ-CEHCs, and α-CEHCs, respectively. CEHC concentrations were calculated from the peak area of the corresponding ion to that of trolox.

Plasma Lipids

Plasma triglycerides and total cholesterol were measured by standard clinical assays (Sigma kit). Prior to study start, plasma triglycerides, HDL, LDL, and total cholesterol (**Table 5**) were measured as part of a blood chemistry panel (Good Samaritan Hospital, Corvallis, OR) that was used to screen potential subjects with abnormal lipid values.

Plasma Antioxidants

Ascorbic acid concentrations were determined by paired-ion reversed-phase HPLC coupled with electro-chemical detection using a modification of the method described by Kutnink et al. (153). Tocopherols were measured using a modified version of the method of Podda et al. (152) using paired-ion reversed-phase HPLC coupled with electro-chemical detection. Plasma α-

tocopherol was standardized for changes in lipoproteins using plasma lipid levels (cholesterol + triglycerides).

Statistical Analyses

Data are expressed as the mean ± SE of 22 subjects. Analysis of variance for repeated measures was used to detect statistically significant differences between and within subject effects (154). An unpaired t-test was used to analyze differences between genders with regard to subject characteristics (i.e. age, height, weight). Statistics were calculated using The SAS System (SAS Institute Inc, Cary, NC).

Vitamin E kinetics were calculated by first dividing the study into three periods: **1)** Pre-race period, 24-48 h following deuterated α-tocopherol consumption (Rate 1), **2)** Race period (Rate 2), and **3)** Recovery period, 6 days post-race (Rate 3; **Figure 19**). The exponential disappearance rates of plasma d_6 (d_6-RRR-α-tocopherol) and d_6/lipids (d_6-RRR-α-tocopherol per lipids (cholesterol + triglycerides)) were assessed for each period using logarithmic transformed data and the linest function of Excel (Microsoft). For rate 2, the time of maximum value on race day was chosen as the initial time point because plasma d_6 concentrations increased initially perhaps as a result of eating.

Table 5. Baseline Plasma Lipids.

Parameter	Women		Men	
	AO (N=6)	PL (N=5)	AO (N=6)	PL (N=5)
Total Cholesterol (mM)	4.7 ± 0.3	4.5 ± 0.4	4.7 ± 0.6	5.0 ± 0.5
LDL Cholesterol (mM)	2.7 ± 0.2	2.6 ± 0.3	2.7 ± 0.5	3.3 ± 0.5
HDL Cholesterol (mM)*	1.6 ± 0.2	1.6 ± 0.1	1.2 ± 0.2	1.2 ± 0.1
Triglycerides (mM)	0.9 ± 0.2	0.8 ± 0.1	1.2 ± 0.2	0.9 ± 0.1

Values are means $\pm$ SE. *men vs women, $p < 0.05$

Figure 19. d_6-*RRR*-α-Tocopherol Concentrations. The subjects in both treatment groups (AO and PL) consumed 75 mg d_6-*RRR* α-tocopheryl acetate 48 hours prior to the race (time 0). Blood samples were obtained the day before the race (24 h pre-race), the evening before the race (12 h pre-race), 1 h prior to the race (pre-race, 0h), in the middle of the race at kilometer 27 (mid-race, 5 h), immediately post-race, 2 h after race end (2 h post-race, 10 h) and for 6 days after the race (post 1-6 days, 24-144 h). All were fasting morning blood draws except 12 h pre-, mid, post- and 2h post-race.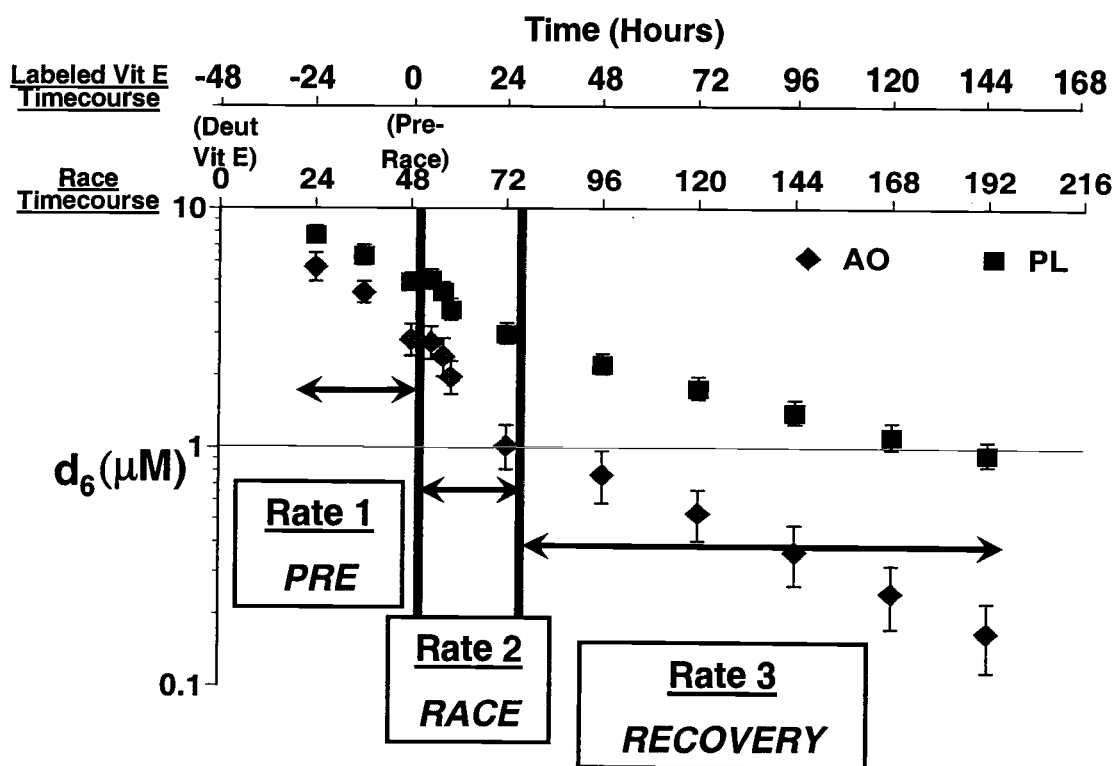 Vitamin E kinetics were calculated for each of three periods: **1)** Pre-race period, 24-48 h following deuterated α-tocopherol consumption (Rate 1), **2)** Race period (Rate 2), and **3)** Recovery period, 6 days post-race (Rate 3). d6 Concentrations were similar in the AO and the PL groups 24 h after consumption of deuterium-labeled vitamin E (24 h prior to the race).

RESULTS

RACE RESULTS

Race results have been reported previously (2, 14). Briefly, all 22 subjects completed the race; only 21 were reported previously (14) due to spurious findings for the comet assay in one subject. Run time averaged 423 ± 11 min at a pace of 13.7 ± 0.4 min/mile and an intensity of 71 ± 2 %$VO_{2\,max}$; there were no statistically significant differences in run time, pace or %VO_{2max} between the genders or the treatment groups. Energy *expenditure* was approximately 2000 kcal greater for men than women and energy *intake* was greater for men than women (Energy Intake (kcal) for men 2530 ± 325 (AO) 2468 ± 279 (PL) compared with 1844 ± 137 (AO) 2040 ± 221 (PL) for women); neither parameter was different between AO and PL groups within each gender. Vitamin E and C intakes during the run were nominal, < 5 mg and < 50 mg respectively; intakes did not differ between treatment groups or genders.

PLASMA ANTIOXIDANTS IN RESPONSE TO SUPPLEMENTATION

In response to 6 w supplementation, as reported previously (2, 14), plasma α-tocopherol increased in the AO supplement group (28 ± 2 vs. 46 ± 3, $p < 0.05$), but was unchanged in the PL group (24 ± 2 vs. 26 ± 2 μM). Similarly, ascorbic acid increased in the AO group (113 ± 14 to 127 ± 12; $p <$ 0.05) but was unchanged in the PL group (93 ± 11 vs. 73 ± 12 μM).

PLASMA LIPIDS

At baseline (~6 w before the race), lipoprotein cholesterol was estimated in fasting blood samples. HDL cholesterol was higher in women than in men ($p < 0.01$, **Table 5**); no other differences in plasma lipids were

observed between genders. There were no differences in plasma lipids between treatment groups within each gender. Compared to pre-race, plasma cholesterol was higher at mid-race (p< 0.04), but was lower 2 h, and 1 and 2 d post-race (p < 0.04, **Figure 20A**). Cholesterol levels were similar in women and men throughout the study. The pattern of changes in plasma triglycerides was similar to those of cholesterol. Compared to pre-race, plasma triglycerides were higher at mid-race (p< 0.002), but lower 2 h, and 1, 2 and 3 d post-race (p < 0.04, **Figure 20B**). Plasma triglycerides were similar in women and men throughout the study.

Figure 20. Plasma Lipids. Compared to pre-race, plasma cholesterol concentrations were increased at mid-race (p< 0.04), but decreased 2 h, and 1 and 2 d post-race (p < 0.04)**(A)**. Compared to pre-race, plasma triglycerides were increased at mid-race (p< 0.002), but decreased 2 h, and 1, 2 and 3 d post-race (p < 0.04)**(B)**.
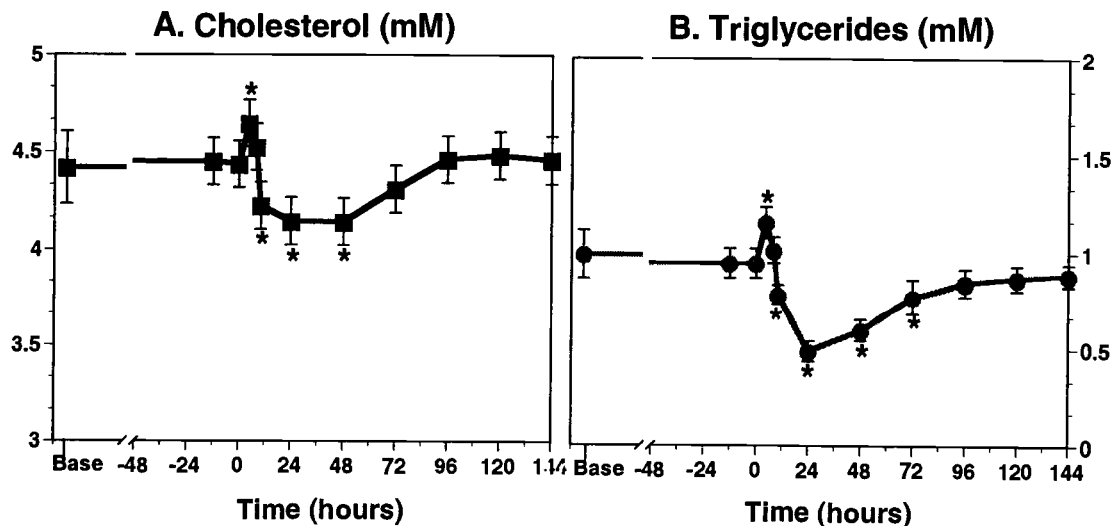
VITAMIN E KINETICS

For kinetic analysis of within subject changes, it was necessary to administer the deuterated vitamin E prior to the race such that the peak plasma concentration would occur before the race. Blood samples were therefore obtained after administration of the labeled vitamin E at 24, 36 and

48 h. The 48 h timepoint is race start; both time scales are shown in Figures 19, 20 and 22.

d_6-RRR-α-Tocopherol Disappearance

Twenty-four h after consumption of deuterium-labeled vitamin E (24 h pre- race), d_6 concentrations were similar in the AO and the PL groups **(Figure 19)**. However, exponential rates of d_6-*RRR*-α-tocopherol disappearance were faster in the AO compared with the PL group for all 3 time periods: pre-race, race, recovery (see Figure 19 for descriptions, Treatment main effect, $p < 0.0001$; **Figure 21**). For all four groups (AO and PL, both genders), d_6 disappearance rates during the run (Rate 2) were faster than both prior to the run (Rate 1)($p < 0.008$) and during recovery (Rate 3)($p < 0.0001$; **Figure 21**). In addition, Rate 1 was faster than rate 3 for all groups ($p < 0.0001$). Unexpectedly, d_6 disappearance rates were faster in women compared with men for all rates (Gender main effect, $p < 0.006$), regardless of treatment.

d_6-RRR-α-Tocopherol/Lipids Disappearance

d_6-*RRR*-α-tocopherol corrected for lipid concentrations (cholesterol plus triglycerides, d_6/lipids) were similar in the AO and PL groups 24 h after consumption of deuterium-labeled vitamin E (24 h pre-race) **(Figure 22)**. Despite similar initial concentrations, d_6/lipids disappearance rates were faster in the AO compared to the PL group for all 3 rates (Treatment main effect, $p < 0.0001$, **Figure 23**). In all 4 groups, pre-race and race period d_6/lipids disappearance rates were similar, while rates during recovery were significantly slower than pre-race ($p < 0.0004$) or during the race ($p < 0.006$; **Figure 23**). All d_6/lipids disappearance rates were faster in women compared with men regardless of treatment (Gender main effect, $p < 0.003$).

Figure 21. d_6-*RRR*-α-Tocopherol Disappearance Rates. d_6-*RRR*-α-Tocopherol disappearance rates were calculated for each individual from the data shown in figure 1. Rates were faster in the AO compared with the PL group at all 3 time periods (Treatment main effect, $p < 0.0001$). For all four groups (AO and PL, both genders), d_6 disappearance rates during the run) were faster than both prior to the run ($p < 0.008$) and during recovery ($p < 0.0001$). Pre-race disappearance rates were faster than recovery disappearance rates for all groups ($p < 0.0001$). d_6 Disappearance rates were faster in women compared to men at all time periods (Gender main effect, $p < 0.006$), regardless of treatment group.
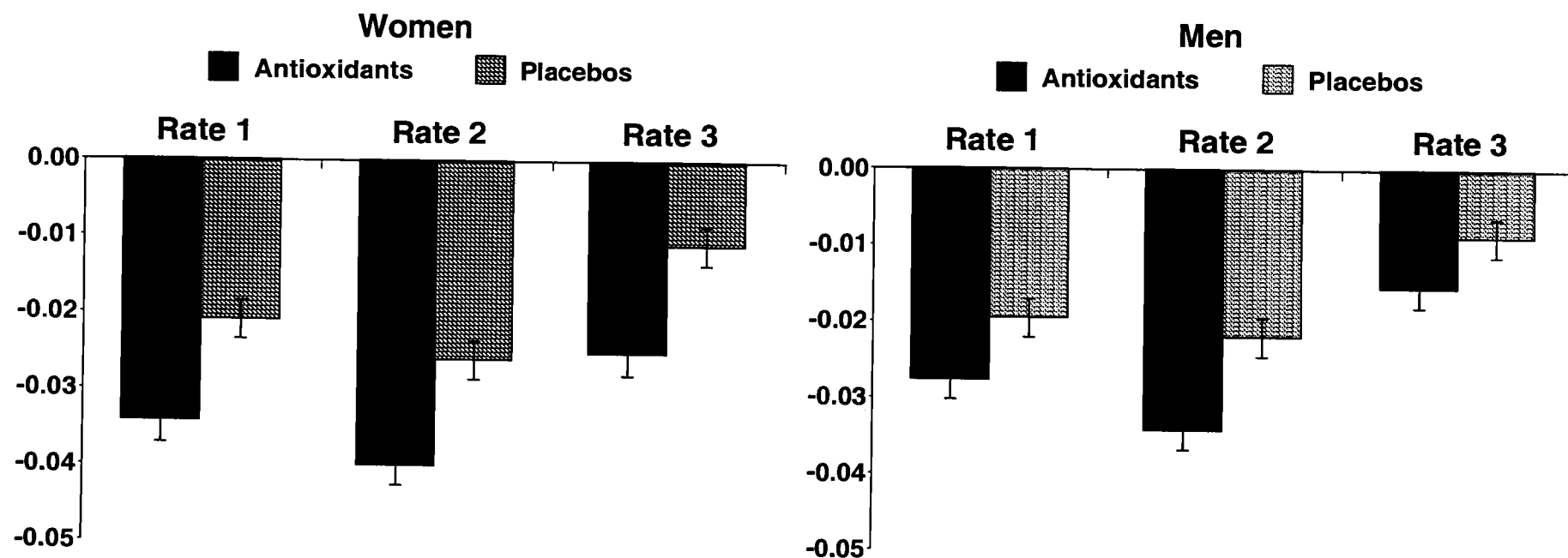
Figure 22. d_6-*RRR*-α-Tocopherol concentrations corrected for lipid concentrations. d_6-*RRR*-α-Tocopherol concentrations corrected for lipid concentrations (cholesterol plus triglycerides, d6/lipids) were similar in the AO and PL groups 24 h after consumption of deuterium-labeled vitamin E (24 h pre-race).
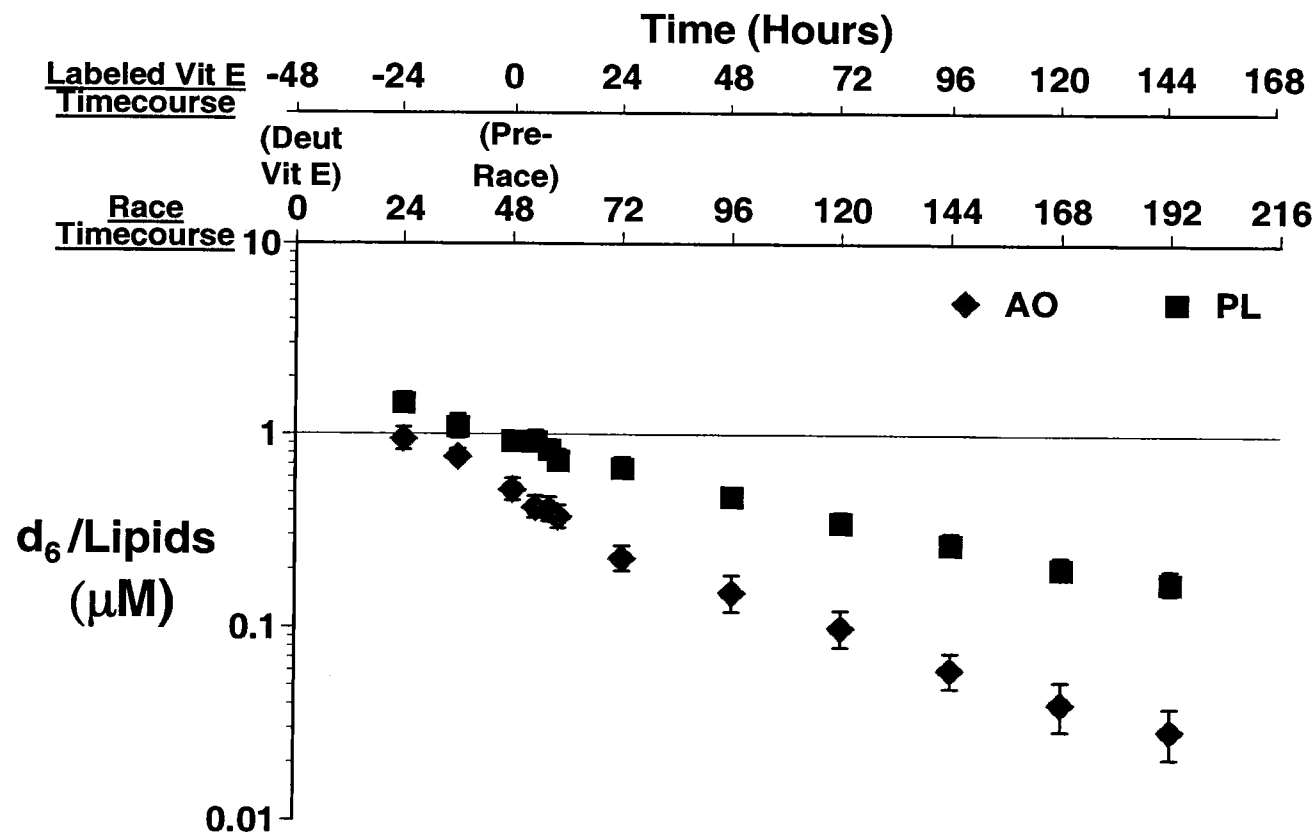
Figure 23. d_6-*RRR*-α-Tocopherol /Lipids Disappearance rates. d_6-*RRR*-α-Tocopherol /Lipids disappearance rates were faster in the AO compared to the PL group for all 3 time periods (Treatment main effect, $p < 0.0001$). In all four groups, pre-race and race period d_6/lipids disappearance rates were similar, while rates during recovery were significantly slower than pre-race ($p < 0.0004$) or during the race ($p < 0.006$). All d_6/lipids disappearance rates were faster in women compared with men regardless of treatment group (Gender main effect, $p < 0.003$).
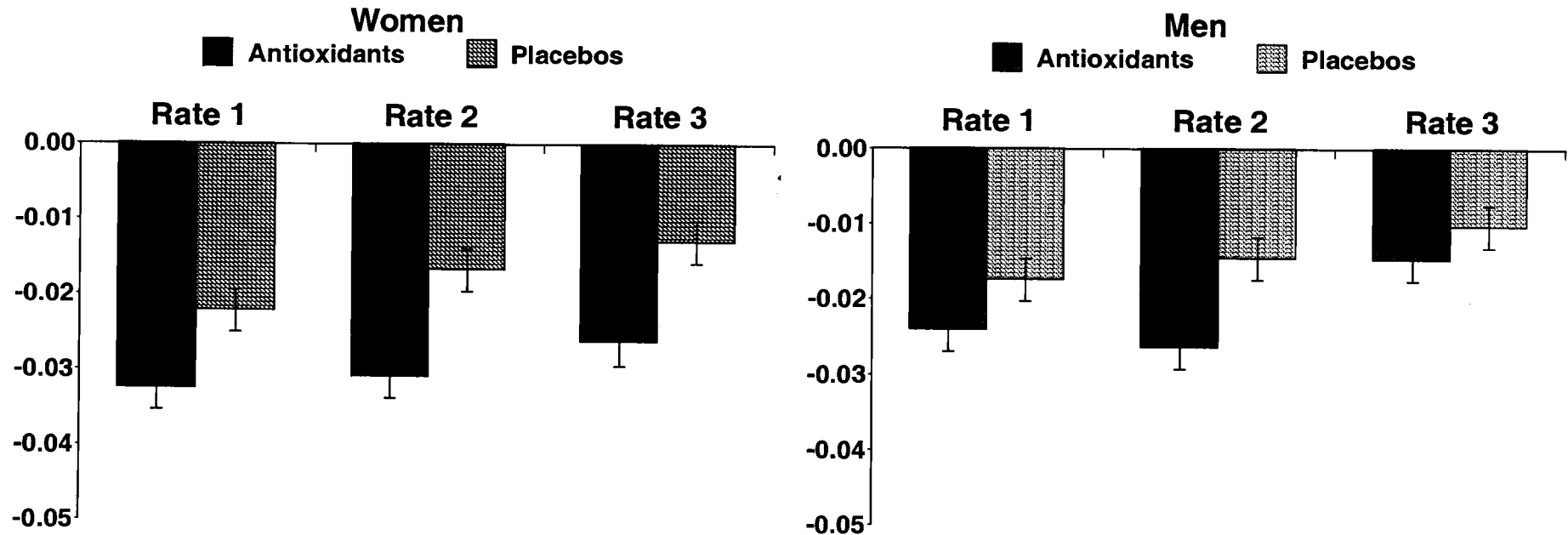
α–CEHC AND γ–CEHC

Faster vitamin E disappearance in the AO groups could be a result of increased vitamin E metabolism; therefore, CEHCs were measured. d_6-CEHC concentrations were below levels of detection in all plasma samples. Following AO supplementation, plasma α-CEHC concentrations were 2 to 3-times those of the PL group at all time points (Treatment main effect, p < 0.0001; **Figure 24**).

Figure 24. Plasma α-CEHC. Following supplementation, plasma α-CEHC concentrations in the AO group were 2 to 3-times than those of the PL group at all subsequent time points (Treatment main effect, p < 0.0001). α-CEHC concentrations changed only in the PL group in response to the run (Treatment X Time interaction, p < 0.002). *Compared to pre-race, α-CEHC concentrations were lower at mid- and post-race (p < 0.007), but returned to pre-race concentrations by 2 h post-race in the PL group.
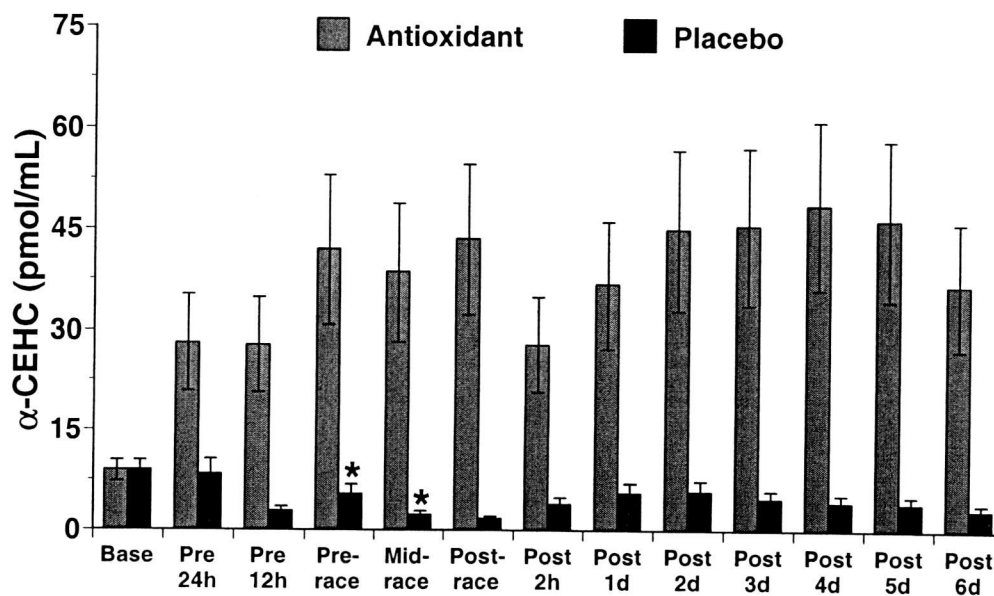
In response to the run, α-CEHC concentrations changed only in the PL group (Treatment X Time interaction, p < 0.002). Compared to pre-race, α-CEHC concentrations were lower at mid- and post-race (p < 0.007), but returned to pre-race concentrations by 2 h post-race in the PL group.

Plasma γ-CEHC concentrations were approximately 10-times higher than baseline γ-CEHC concentrations, but were not influenced by AO supplementation or by the ultramarathon run (**Figure 25**).

Figure 25. Plasma γ-CEHC. Plasma γ-CEHC concentrations were not influenced by AO supplementation or by the ultramarathon run.
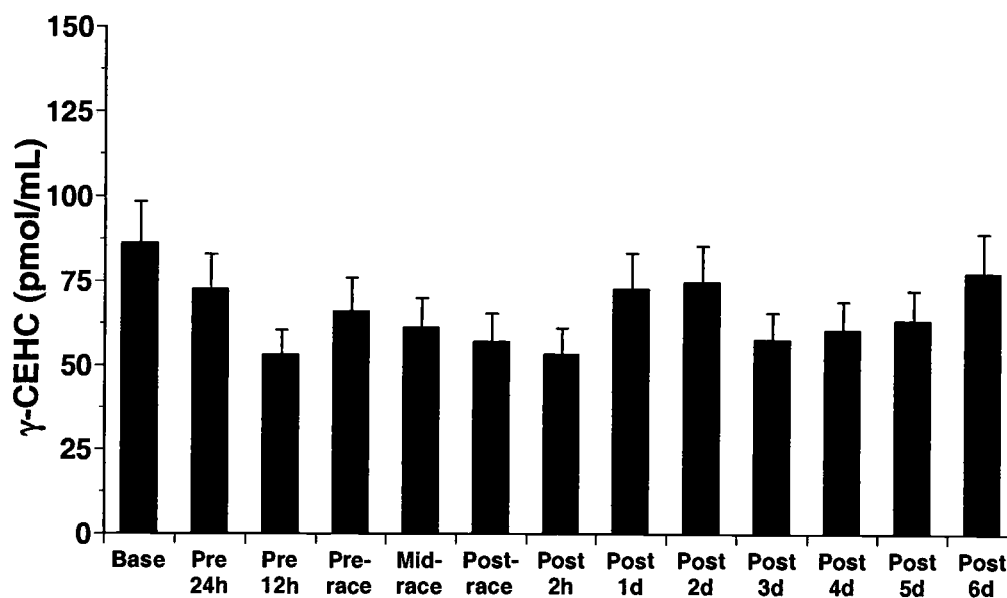
DISCUSSION

We observed faster d_6-*RRR*-α-tocopherol disappearance rates during the run compared to the preceding rest day (**Figure 21**) consistent with our previous findings that endurance exercise increases disappearance of deuterium labeled α-tocopherol (1). Furthermore, d_6 disappearance rates were faster during the race compared with the recovery period for all four groups (**Figure 21**). These findings are consistent with a multicompartmental model of vitamin E kinetics with faster and slower compartments (93) with a slower d_6 disappearance 72 h after the dose (Traber unpublished).

We have also reported (2) that in these runners the PL compared with the AO groups experienced more oxidative stress following running as evidenced by increased F_2-IsoPs. Consequently, it was an unexpected finding that d_6 disappearance rates were faster in the AO compared with the PL group. It was also unexpected that d_6 disappearance rates were faster in women than men, regardless of treatment group.

Plasma tocopherols are transported entirely within lipoproteins and fluctuate with lipoprotein concentrations (178). Due to this relationship between α-tocopherol concentrations and plasma lipids, we asked the question: Are the observed differences in d_6 disappearance rates explained by fluctuations in lipid levels with exercise? Plasma lipids (total cholesterol and triglycerides) were elevated in the middle of the race, but decreased to below pre-race levels 2 h post-race and remained low up to 2 (cholesterol) and 3 (triglycerides) days post-race (**Figure 20**). They did not differ between treatment groups or genders over the course of the study. Correction of $d_6\,\alpha$-tocopherol concentrations for fluctuations in lipid levels (cholesterol +

triglycerides) yielded somewhat different kinetics than d_6 concentrations alone. Like d_6, disappearance of d_6/lipids was faster in the AO compared to the PL group at all time periods and disappearance of d_6/lipids was faster in women compared to men regardless of treatment for all 3 rates. However, rates of d_6/lipids disappearance were similar during the pre-race and race periods for all 4 groups. These findings suggest that increased lipoprotein turnover accounts for the faster d_6-α-tocopherol disappearance observed during the race compared to pre-race. Furthermore, the higher HDL concentrations in women compared to men (Table 5) suggest that HDL delivery to the liver may be important in plasma vitamin E clearance. Studies in scavenger receptor-BI (SR-BI) deficient mice have demonstrated that selective uptake of HDL lipids and α-tocopherol via the SR-B1 facilitates biliary α-tocopherol excretion. Thus, women with their higher HDL levels may clear plasma vitamin E faster than do men. However, studies of SR-B1 regulation by vitamin E in type II pneumocytes (179) or of scavenger receptor family member, CD36 (180) in macrophages (181) or smooth muscle cells (182), have all demonstrated that α-tocopherol down-regulates expression of scavenger receptors. Thus, increased lipoprotein turnover cannot explain the faster disappearance rates in the AO groups because the supplemental vitamin E should have *decreased* SR-BI expression. Certainly, additional studies examining liver SR-B1 regulation by α-tocopherol are needed to explore this point.

A potential mechanism for the increased plasma d_6 disappearance in the AO groups could be explained by increases in non-oxidative metabolism of α-tocopherol to CEHC. Recent studies examining α-tocopherol binding to the nuclear receptor PXR (183) suggest that excess hepatic α-tocopherol may up-

regulate its own metabolism (184). However, we found that circulating $d_6\alpha$-CEHC concentrations were below our levels of detection. Nonetheless, AO supplementation increased unlabeled α-CEHC concentrations 5-10 fold compared to baseline or to levels in the PL group. No differences in γ-CEHC were observed between treatment groups or genders.

No changes in α-CEHC were observed in the AO group in response to the run. α-CEHC concentrations decreased in the PL group at mid-race and post-race suggesting that less α-tocopherol was available to be converted into the metabolite. No gender differences in α-CEHC were observed at any time during the study and therefore increased metabolism of α-tocopherol to α-CEHC can not explain increased rate of d_6 and d_6/lipids disappearance in women compared to men. Since we were not able to measure bile, we have no way of determining how much of the d_6 was excreted by this route.

Recently, we reported that in these runners vitamins E and C were protective against exercise-induced lipid peroxidation as evidenced by increased F_2-IsoPs in the PL but not the AO group, following the ultramarathon (2). Due to this observed increase in lipid peroxidation, we anticipated that α-tocopherol utilization (d_6 disappearance) during the run would be increased in the PL, but not the AO group. Instead, we observed a faster rate of d_6 disappearance in the AO group throughout the study time course including the race. One explanation is that metabolism of α-tocopherol by cytochrome p450s in the liver is up-regulated in response to vitamin E supplementation resulting in increased α-tocopherol disappearance independent of the exercise bout. This hypothesis is supported by the higher α-CEHC levels in the AO group throughout the supplementation period and the similar rates of disappearance of d_6/lipids before *and* during the run.

Following the race, elevated plasma F_2-IsoPs concentrations in the PL women returned rapidly to pre-race concentrations, but remained elevated in the PL men (2). This gender difference may be explained by differences in α-tocopherol utilization (d_6 disappearance) as disappearance of d_6 and d_6/lipids was faster in PL women than PL men both during the race and during recovery. These data suggest that women may have been more protected against oxidative stress due to an increased ability to utilize vitamin E and therefore able to recover at a faster rate.

Increased disappearance of d_6 during exercise compared to rest, in women compared to men and in AO supplemented versus PL are interesting and novel findings. Nonetheless, results in the present study reflect *plasma* vitamin E kinetics and may or may not be representative of vitamin E turnover in the active muscle. Therefore, it seems likely that studies that undertake muscle biopsies are necessary in order to gain insight into the relationship of vitamin E kinetics and oxidative stress in the working muscle.

CHAPTER 5: ANTIOXIDANTS DO NOT PREVENT MUSCLE DAMAGE OR FATIGUE IN RESPONSE TO AN ULTRAMARATHON RUN

Angela Mastaloudis[1,2], Jeff Widrick[1], Kristen Carstensen[1]
and Maret G. Traber[1,2,3]

[1]Department of Exercise and Sport Science, Oregon State University, Corvallis, OR; [2]Linus Pauling Institute, Corvallis, OR; [3]Department of Nutrition and Food Management, Oregon State University, Corvallis, OR

Antioxidants Do Not Prevent Muscle Damage or Fatigue in Response to an Ultramarathon Run

ABSTRACT

To determine if 6 w supplementation with vitamins E and C could alleviate exercise-induced muscle damage, we studied 22 runners during a 50 km ultramarathon. Subjects were randomly assigned to one of two groups: 1) placebos (PL) or 2) antioxidants (AO) (1000 mg vitamin C and 400 IU RRR-α-tocopheryl acetate). Blood samples were obtained prior to supplementation (baseline), 24 h pre-, 12 h pre-, and 1 h pre-race, mid-race, post-race, 2 h post-race and for six days post-race. Plasma α-tocopherol (α-TOH), ascorbic acid (AA), and muscle damage markers (creatine kinase (CK) and lactate dehydrogenase (LDH)), as well as maximal voluntary contraction (MVC) of the hamstring and quadriceps were assessed. With supplementation, plasma α-TOH and AA increased in the AO but not the PL group. In response to the run, MVC ($p < 0.0001$) decreased similarly in all groups. The largest force deficit (23%) was observed for the eccentric hamstring contraction, while eccentric quadriceps decreased 16%, concentric hamstring 14%, and concentric quadriceps 17%. Eccentric hamstring contraction force had not recovered by study end. No differences between genders or treatment groups were observed for MVC force deficit. LDH and CK increased in response to the race (Time main effects, $p < 0.0001$ for each); neither was affected by gender or treatment. CK was significantly correlated ($R=0.52$, $p < 0.0001$) with C-reactive protein, an acute phase response marker. Antioxidants appeared to have no effect on exercise-induced increases in muscle damage or fatigue.

INTRODUCTION

Endurance running results in muscular damage and fatigue associated with ultrastructural changes (185), increased release of muscular enzymes into the plasma (185) and substantial impairment in maximal force production (186). While these effects may be the result of mechanical muscle damage, evidence that oxidative damage by reactive oxygen species (ROS) mediates skeletal muscle damage is accumulating (43, 185).

It is generally accepted that at rest the body produces continuously. In healthy individuals at rest, these ROS are produced at levels well within the capacity of the body's antioxidant defense system. In response to endurance exercise, oxygen (O_2) consumption increases 10 to 20-fold systemically and as much as 100 to 200-fold at the level of the skeletal muscle (23) resulting in substantially increased mitochondrial electron flux (6). Reactive oxygen species (ROS) leaking from the mitochondria during exercise are considered a main source of oxidative stress (23) as oxidant production outpaces antioxidant defenses. Other potential sources of sources of ROS during exercise include enhanced purine oxidation, damage to iron-containing proteins, disruption of Ca^{2+} homeostasis (24), neutrophil activation (8) and autoxidation of catecholamines (3).

Vigorous exercise results in oxidative stress (21) as evidenced by increased lipid peroxidation (1), DNA damage (14) and protein oxidation (53). Damage to skeletal muscle cell membranes by ROS, specifically lipid peroxidation, can impair cell viability leading to necrosis and an acute phase inflammatory response (25, 43). Quenching of ROS by antioxidants could protect against muscle damage caused by exercise. Therefore, the purpose

of the present study was to determine if prior supplementation with the antioxidant vitamins E and C could attenuate exercise-induced muscle damage and/or accelerate the rate of recovery from damaging exercise.

EXPERIMENTAL PROCEDURES

HUMAN SUBJECTS

A description of the study design has been published previously (2, 14). Briefly, the protocol for this study was approved by the Oregon State University Institutional Review Board for the Protection of Human Subjects. Runners (11 women and 11 men) were recruited from the pool of participants in a 50 km (32 mile) ultramarathon trail run.

The physical characteristics of the subjects have been reported previously (14). Average age was 39 ± 2.5 yrs, VO_{2max} 58 ± 1 ml/kg/min and estimated weekly training distance 43 ± 3 km; there were no differences between treatment groups or genders for these characteristics. Men were taller, weighed more and had lower percent body fat than women. Women in the AO group weighed slightly less than women in the PL group ($p < 0.05$) while men in the AO group were slightly taller than men in the PL group ($p < 0.05$). Within each gender, VO_{2max} and % body fat did not differ among treatment groups. During training, estimated average daily nutrient intakes were 2653 ± 201 kcal, 142 ± 26 mg vitamin C and 14 ± 3 mg vitamin E; there were no differences between men and women in energy, dietary vitamin E or C intakes.

Criteria for Subject Participation

Inclusion criteria for participation in the study included non-smoking status, age 18-60 y, and a VO_{2max} classified as excellent fitness by Powers

and Howley (45). Potential participants were excluded based on antioxidant supplement use (e.g. vitamin C, vitamin E, selenium or carotenoids), abnormal cholesterol ($\geq$ 7.8 mmol/L (300 mg/dl)), triglyceride ($\geq$ 3.8 mmol/L (300 mg/dl)) or fasting blood glucose levels ($\geq$ 7.8 mmol/L (140 mg/dl)) or other supplement use (performance enhancing, or herbal type products), vegetarian or other restrictive dietary requirements, pregnancy or suspected pregnancy, and chronic upper respiratory infections.

STUDY DESIGN

Randomization to Treatment Group

Subjects were randomly assigned in a double-blind fashion to one of two treatment groups 1) PL (300 mg soybean oil and 1000 mg citric acid (500 mg twice daily) or 2) AO (300 mg *RRR*-α-tocopheryl acetate [2,5,7,8-tetramethyl-2*R*-(4'*R*,8'*R*,12-trimethyltridecyl)-6-chromanol] in soybean oil and 1000 mg ascorbic acid (500 mg twice daily)).

Muscle Function Assessment

Maximal voluntary contraction (MVC) of the knee extensors/flexors (quadriceps/hamstrings) was measured using a KinCom isokinetic dynamometer. Subjects were tested at baseline (prior to supplementation), the day prior to the race (1 day pre-race), 2 h after the race (2 h post-race) and for six days following the race for a total of 9 testing sessions. After a demonstration of the procedures, subjects were seated and secured in the rigid seat of the dynamometer in order to isolate the left knee extensor and flexor muscle groups. Subjects were instructed to perform ~10 contractions well below their maximum as a warm-up. After the warm-up, subjects performed 3 maximal concentric knee extensions and flexions followed by 3

maximal eccentric knee extensions and flexions at a velocity of 60°/sec; peak voluntary torque (MVC) was recorded for each contraction. Subjects were given a one-minute rest between the concentric and eccentric measurements. The left leg was used for all sessions.

Blood Samples

Blood samples were obtained prior to antioxidant or placebo supplementation (baseline), the day before the race (24 h pre-race), 1 h prior to the race (pre-race (0h)), in the middle of the race at kilometer 27 (mid-race (5 h)), immediately post-race, 2 h after race end (2 h post-race (10 h)) and for six days after the race (post 1-6 days (24-144 h)), for a total of twelve time points. All samples were fasting morning blood draws except mid-, post- and 2h post-race.

ANALYTICAL METHODS

Muscle Damage Markers

Plasma creatine kinase and lactate dehydrogenase were measured by standard clinical assays (Sigma kit, St. Louis, MO).

Plasma Antioxidants

Ascorbic acid was determined by paired-ion reversed-phase HPLC coupled with electro-chemical detection using a modification of the method described by Kutnink et al. (153). α-Tocopherol was determined by paired-ion reversed-phase HPLC coupled with electro-chemical detection using a modified version of the method of Podda et al. (152).

Statistical Analyses

Data are expressed as the mean $\pm$ SE of 22 subjects. Analysis of covariance for repeated measures was used to detect statistically significant

between and within subject effects (154). In order to adjust for pre-existing differences between individuals prior to supplementation, baseline concentrations of the following markers were used as covariates in the corresponding statistical model. Baseline covariates included: CK ($p < 0.003$), LDH ($p < 0.0001$) and MVC; eccentric hamstring ($p < 0.0009$), concentric hamstring ($p < 0.007$), eccentric quadriceps ($p < 0.0001$) and concentric quadriceps ($p < 0.04$). An unpaired t-test was used to analyze differences between genders with regard to subject characteristics (i.e. age, height, weight). Statistics were calculated using The SAS System (SAS Institute Inc, Cary, NC).

RESULTS

RACE RESULTS

Race results have been reported previously (2, 14). Briefly, all 22 subjects completed the race; previously only 21 were reported due to spurious findings for the comet assay in one subject (14). Run time averaged 423 ± 11 min at a pace of 13.7 ± 0.4 min/mile and an intensity of 71 ± 2 %$VO_{2\,max}$; there were no statistically significant differences in run time, pace or %VO_{2max} between the genders or the treatment groups. Energy *expenditure* was approximately 2000 kcal greater for men than women and energy *intake* was greater for men than women (Energy Intake (kcal) 2530 ± 325 (AO) 2468 ± 279 (PL) for men compared with 1844 ± 137 (AO) 2040 ± 221 (PL) for women); neither parameter was different between AO and PL groups within each gender. Vitamin E and C intakes during the run were nominal, < 5 mg and < 50 mg respectively; intakes did not differ between treatment groups or genders.

PLASMA ANTIOXIDANTS IN RESPONSE TO SUPPLEMENTATION

In response to 6 w supplementation, as reported previously (14), plasma α-tocopherol increased in the AO supplement group (28 ± 2 vs. 46 ± 3, $p < 0.05$), but was unchanged in the PL group (24 ± 2 vs 26 ± 2 μM). Similarly, ascorbic acid increased in the AO group (113 ± 14 to 127 ± 12; $p < 0.05$) but was unchanged in the PL group (93 ± 11 vs 73 ± 12 μM).

MAXIMAL VOLUNTARY CONTRACTION: FATIGUE AND RECOVERY

The MVC expressed as maximal peak torque (Nm) of each of the 3 contractions for each test was corrected for subjects' lean body mass and used to determine force deficit and recovery in response to the run. In response to the run, MVC decreased in all muscle groups in both AO and PL groups ($p < 0.0001$, **Figure 26**). The largest force deficit, 23%, was observed for eccentric hamstring (**Figure 26A**). Other force deficits: concentric hamstring 14%, eccentric quadriceps 16% and concentric quadriceps 17%, demonstrated that the exercise decreased force similarly in these three muscle contractions (**Figure 26B-D**). In addition to exhibiting the largest decline in MVC, the eccentric hamstring values remained below pre-race values for the duration of the study (**Figure 26A**). In contrast, concentric hamstring, eccentric and concentric quadriceps had recovered to pre-race values by 4 days post-race (**Figure 26B-D**). Concentric hamstring reached higher levels than pre-race at 6 days post-race ($p < 0.02$, **Figure 26B**). No differences between genders or treatment groups were observed for any parameter.

Figure 26. Muscle Force Deficit. The maximal peak torque in Nm of each of the 3 maximal contractions for each test was corrected for subjects' lean body mass. MVC decreased similarly in all groups in response to the run (p < 0.0001). Eccentric hamstring, -23% force deficit, did not recover by end of study **(A)**. Concentric hamstring, -14% force deficit, recovered by 3 days post-race **(B)**. Eccentric quadriceps, -16% force deficit, recovered by 3 days post-race **(C)**. Concentric quadriceps, -17% force deficit, recovered by 3 days post-race **(D)**. No differences between genders or treatment groups were observed. * = compared to pre-race.
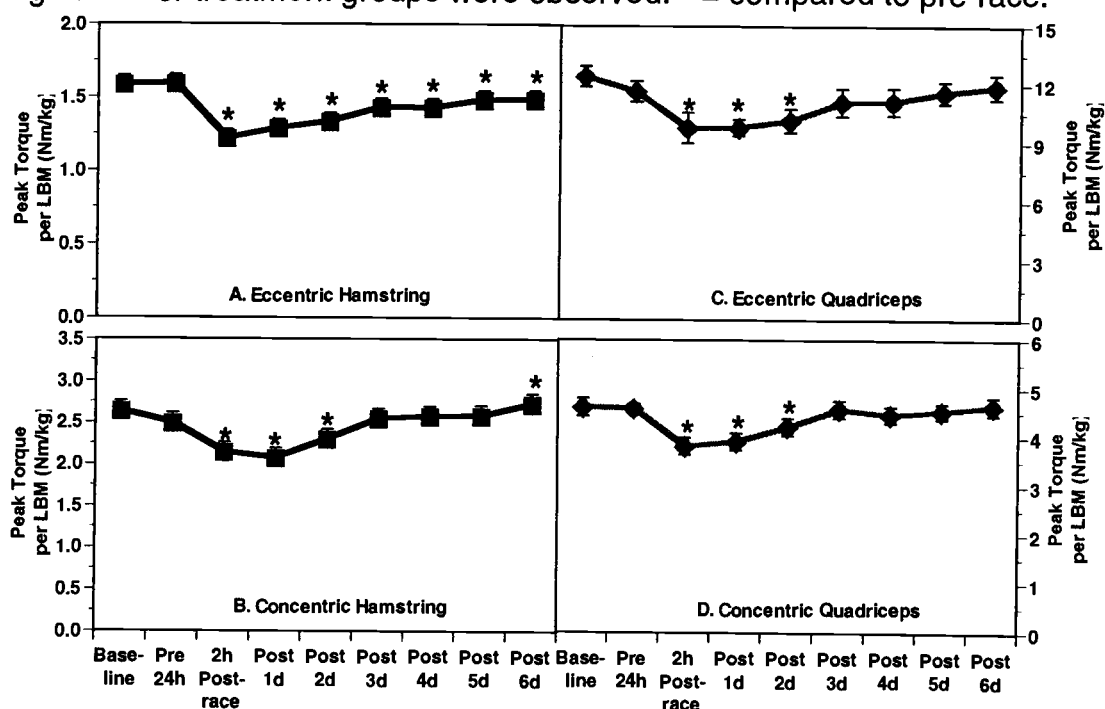
PLASMA MUSCLE DAMAGE MARKERS

LDH increased in response to the race (Time main effect, p < 0.0001, **Figure 27A**) and was unaffected by gender or treatment. Compared to pre-race, LDH increased at mid-race, peaked at post-race (p < 0.01), and remained elevated throughout the recovery period. Similarly, CK increased in response to the race (Time main effect, p < 0.0001, **Figure 27B**). Compared to pre-race, CK increased at mid-race, continued to increase peaking 2 day post-race (p < 0.0001), and remained elevated throughout the recovery period.

Over the course of the study, muscle damage markers CK and LDH were significantly correlated (Pearson product moment correlation R=0.64, p < 0.0001). Furthermore, CK was significantly correlated with c-reactive protein (CRP)(Pearson product moment correlation R=0.52, p < 0.0001). During the period of peak damage (mid-race to post 1 day), similar correlations were observed (CK with LDH, Pearson product moment correlation R=0.64, p < 0.0001; and CK with CRP, Pearson product moment correlation R=0.63, p < 0.0001).

Figure 27. Muscle Damage Markers. Lactate dehydrogenase (LDH) increased in response to the race (Time main effect, p < 0.0001) and was unaffected by gender or treatment. Compared to pre-race, LDH increased at mid-race, peaked at post-race (p < 0.01), and remained elevated throughout the recovery period **(A)**. Creatine kinase (CK) increased in response to the race (Time main effect, p < 0.0001). Compared to pre-race, CK increased at mid-race, continued to increase peaking 2 day post-race (p < 0.0001), and remained elevated throughout the recovery period **(B)**. * = compared to pre-race; # = maximal concentration.
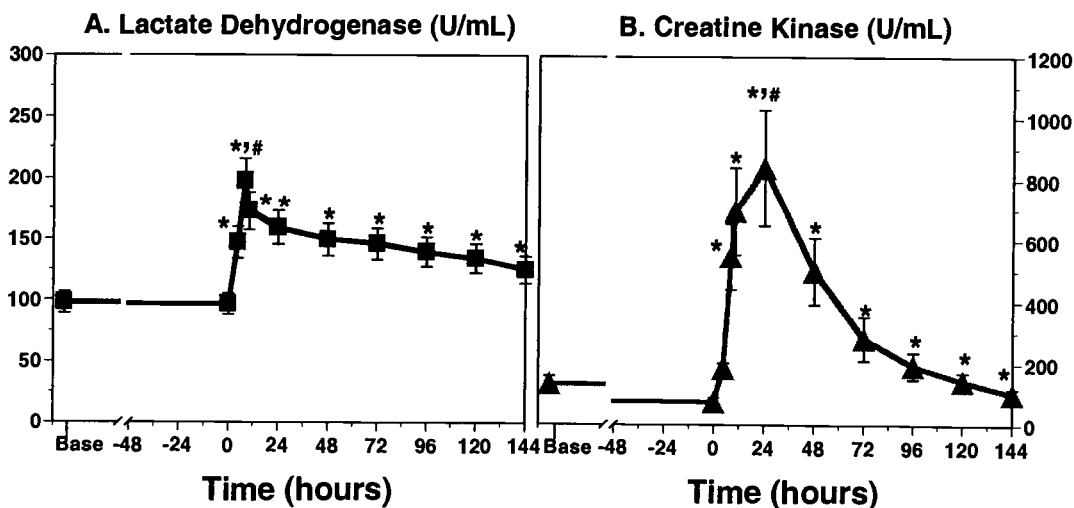
DISCUSSION

The ultramarathon resulted in substantial increases in muscle damage markers and deficits in maximal force production of the knee extensors and flexors. Prior supplementation with the antioxidant vitamins E and C appeared to have no effect on these parameters of muscle damage and fatigue.

The inability of antioxidant supplementation to attenuate exercise-induced muscle damage is in contrast to our previous findings that supplementation completely prevented exercise-induced lipid peroxidation (2), suggesting that muscle damage may be independent of oxidative damage. Muscle damage may be more closely related to inflammation, as CK was significantly correlated with the acute phase protein C-reactive protein in the present study. Previously we reported that the inflammatory and lipid peroxidation responses to ultramarathon running appear to be unrelated (2).

Alternatively, the ultramarathon run may have been too strenuous and therefore the oxidative stress overwhelmed the protective effects of the antioxidants. Previously vitamin C was found to be protective in a more moderate exercise protocol, 60 minutes of box-stepping exercise (187). One w of prior supplementation with 400 mg vitamin C enhanced the rate of recovery of MVC force deficit (187). In this same study, supplementation with 400 mg vitamin E had no effect on recovery, however, plasma vitamin E was not substantially increased in the short supplementation period (3 w prior to and 1 w post exercise) (187). When vitamin E was supplemented for a longer time period at a higher dose (1200 IU for 3 w) and plasma vitamin E levels were more than doubled, supplementation still had no effect on exercise-

induced concentric force deficits following a short muscle-damaging exercise protocol (188).

CK is the most commonly used marker of muscle damage; it is well documented that CK increases in response to marathon running, reaching peak levels 24 h post-race in trained runners (61). In the present study, CK was elevated at post-race, but did not reach maximal concentrations until 24 h post-race consistent with previous reports (61). Study of the response of CK to antioxidant supplementation has yielded equivocal results. α-Tocopherol supplementation (1200 IU/day for 4 w) attenuated CK increases following 6 successive days of running (129), but no effect of 13.5 mg/day for 3 w prior to a marathon run on the plasma CK response was observed (110). Vitamin C supplementation as a single 1000 mg dose 1 hour prior to a 4 hour run did not attenuate CK increases (33), but supplementation with 1000 mg/day vitamin C for 1 w prior to a 90 km run exacerbated the CK and CRP response to exercise suggesting an adverse effect of supplementation (111). Our observation that combined supplementation with 300 mg vitamin E and 1000 mg vitamin C had no effect on exercise-related increases in CK is in agreement with the study of Petersen et al. (107). They reported no influence of 2 w supplementation with 400 mg vitamin E and 500 mg vitamin C on increases in CK following a 90 minute treadmill run (107). Our findings are in contrast, however, to those of Rokitzki et al. (13), who reported that 4.5 w supplementation with 400 IU vitamin E and 200 mg vitamin C attenuated increases in CK following a 90 km ultramarathon. Thus, results appear to be influenced by amount and duration of dose and the type of antioxidant supplemented, as well as intensity, duration and type of exercise.

A second, less common, plasma marker of muscle damage, LDH, increases in response to marathon running (189). Few studies have investigated the ability of antioxidant supplementation to modulate LDH responses to marathon running. Presently, we report that supplementation with vitamins E and C had no effect on LDH increases following distance running. This finding differs from the report of Itoh et al. (129) that supplementation with 1200 IU/day α-tocopherol for 4 w attenuated LDH increases following 6 successive days of running, suggesting that a larger dose of vitamin E is required to attenuate muscle damage resulting from endurance exercise. Clearly further research is needed to confirm this hypothesis.

In the present study, antioxidants appeared to have no effect on exercise-induced increases in muscle damage, fatigue or recovery from adverse consequences resulting from a marathon run. Future studies may take advantage of muscle biopsy techniques in order to examine the effects of antioxidant supplementation directly at the level of the skeletal muscle.

CHAPTER 6: DISCUSSION

The 50 km ultramarathon run elicited oxidative stress, muscle damage and inflammation. Oxidative stress was demonstrated by increased lipid peroxidation, DNA damage and vitamin E utilization. Muscle damage was characterized by increases in fatigue, as well as increases in circulating muscle damage markers. The changes in circulating inflammatory markers elicited by the run demonstrated the characteristic progression of cytokine responses to damage and inflammation. Overall, the ultramarathon runners provided an ideal model to test the hypothesis that antioxidant supplementation would alleviate the adverse consequences of running. Supplementation with vitamins E and C completely inhibited exercise-induced lipid peroxidation and appeared to benefit women with respect to DNA damage during recovery. Surprisingly, supplementation had no effect on exercise-induced inflammation. Similarly, antioxidants appeared to have no effect on muscle damage markers or on fatigue. These results suggest that oxidative damage may be operating independently of the inflammatory and muscle damage response pathways.

DNA DAMAGE

The exercise bout was sufficiently strenuous that by mid-race, subjects exhibited DNA damage, as assessed using the comet assay (14). However, the proportion of cells with DNA damage returned to baseline values by the end of the race and by 2 days after the race, the proportion of cells with damage declined below baseline values and decreased to 8% below baseline by 6 days post-race (14). The precise reason for this apparent decrease is unclear but may have been due to increased repair mechanisms, increased

clearance of damaged cells, a redistribution of damaged cells and/or because subjects were no longer exercising.

Both men and women within each treatment group had similar circulating antioxidant levels, but women runners had higher levels of DNA damage. Moreover, antioxidant supplementation protected the women runners by decreasing the proportion of cells with damage on the day following the ultramarathon race, while men experienced little benefit from the antioxidants.

There may be benefits to the observed exercise-induced oxidative damage such as, enhanced antioxidant enzyme defenses and/or increased clearance of oxidized LDL particles (123). Relatively low levels of DNA damage generated by exercise may be beneficial by upregulating DNA damage repair enzymes, stimulating the immune response and/or by increasing clearance of damaged cells by inducing apoptosis (162). This concept that low levels of a damaging entity can upregulate protective mechanisms in an organism is known as hormesis (162). In support of this theory, both cohort and case-control epidemiological studies have demonstrated dose-dependent protection of physical activity against overall and site-specific cancer risk (163) and there is little evidence that the exercise-induced DNA damage is mutagenic (137). In fact, Hartmann et al. (15) reported that despite substantial increases in DNA damage as measured by the comet assay in response to a 2.5 h triathlon competition, no increases in frequency of micronuclei, a sensitive indicator of chromosome damage, were observed.

Overall, exercise appears to induce a temporary increase in DNA damage. This increase, however, does not appear to have adverse effects, and may be beneficial because it appears to induce removal of damaged cells.

LIPID PEROXIDATION

Lipid peroxidation increased in the PL group in response to the race, but prior supplementation with vitamins E and C completely inhibited the increase in lipid peroxidation (2). At post-race, when oxidative stress was maximal, F_2-IsoP concentrations were inversely correlated both with α-tocopherol/lipids (R= -0.61, p<0.003) and ascorbic acid (R= -0.41, p= 0.05) (2). These findings provide further documentation that antioxidants were responsible for preventing lipid peroxidation.

Both men and women responded to the run with similar degrees of lipid peroxidation; however responses in the placebo groups during recovery were markedly different. F_2-IsoP concentrations in PL women returned to baseline within 2 hours post-race, whereas higher levels persisted in PL men for the duration of the study. Jenkins and Goldfarb (56) also reported that in response to exercise men experience greater oxidative stress than do women. These data suggest that men may be more susceptible to oxidative stress-induced chronic disease, as well.

We also found that cardiovascular fitness was inversely related to lipid peroxidation based on the inverse correlation between VO_{2max} and baseline circulating F_2-IsoP concentrations (R = -0.55, p < 0.01) (2), confirming previous observations by Liu et al. (49). Moreover, we found that F_2-IsoPs increased only after subjects experienced an exceptional oxidative insult, the ultramarathon, and then only in the PL group. The source(s) of the F_2-IsoPs are unknown. The resumption of weight bearing activity following space flight

caused muscle damage and increased urinary excretion of F_2-IsoPs, implicating a relationship between oxidative stress and muscle damage (120). However, this link between muscle damage and lipid peroxidation was not supported by our findings.

INFLAMMATION

Very few studies have examined the effects of antioxidants on both exercise-induced oxidative stress and inflammation (37, 41). We found that supplementation of runners with vitamins E and C had no effect on TNF-α, IL-6, CRP, IL-1 or ferritin, despite the finding that increases in lipid peroxidation were prevented (2). Nieman et al. (37) reported that ascorbic acid (1500 mg/day) for one w prior to an ultramarathon did not prevent exercise-induced increases in plasma F_2-IsoPs, lipid hydroperoxides or IL-6 (pro-inflammatory cytokine). Childs et al. (41) studied the effects of 1 w *post-exercise* supplementation with ascorbic acid and N-acetyl-cysteine (NAC) on muscle damage. Both F_2-IsoPs and lipid hydroperoxides increased in response to muscle damage, but levels were higher in the *supplemented* group post-exercise. Childs et al. (41) suggested a pro-oxidant effect of ascorbic acid and NAC. Nonetheless, these supplements had no effect on exercise-induced IL-6 increases. In the present study, IL-6 increases in response to the ultramarathon were not modulated by vitamin E and C supplementation (2). Similarly, in the present study , CRP was not attenuated by AO supplementation (2), in accordance with previous reports (170).

ENDOGENOUS MECHANISMS TO INCREASE ANTIOXIDANT DEFENSES

Although the purpose of the present study was to evaluate the effectiveness of supplemental antioxidants in alleviating exercise-induced oxidative damage, some endogenous antioxidant defense responses were

detected. During the 50 km ultramarathon run, plasma ascorbic acid *increased* similarly in both treatment groups with significant increases compared to pre-race, at mid-race and post-race returning to pre-race values by 2 h post-race. Increases in plasma ascorbic acid in response to vigorous exercise have been reported in some (1, 7, 13, 48, 107, 110), but not all (31, 111, 112) studies. Gleeson et al. (115) suggested that exercise-related increases in circulating cortisol promote efflux of ascorbic acid from the adrenal gland and/or the mobilization of ascorbic acid from other tissue sites such as leukocytes or erythrocytes. Some (111, 112, 116), but not all (37), recent studies have demonstrated an attenuation of the exercise related increase in circulating cortisol with vitamin C supplementation. Taken together, these findings suggest that oxidative stress may regulate cortisol secretion.

Plasma uric acid concentrations also increased in response to the run, consistent with the findings of others (13, 49, 100). The increase may be explained by enhanced purine oxidation with exercise (13, 49, 100). Concurrent increases in plasma ascorbic and uric acids may reflect enhanced antioxidant defenses in response to the oxidative stress of the endurance exercise. Moreover, our findings are in accordance with previous studies reporting increased antioxidant enzymes (11) and antioxidant nutrients (6, 13, 48) in response to extreme exercise.

A few of the studies investigating the effects of vitamins E and/or C supplementation on endurance running have reported increases in plasma α-tocopherol concentrations in both supplemented and placebo groups following exercise (13, 40, 103), but most failed to report post-exercise plasma α-tocopherol concentrations (31, 32, 104-106). One study reported no change

in α-tocopherol concentrations with exercise (107). We observed an increase in plasma α-tocopherol concentrations during exercise in the AO, but not the PL group. After correcting α-tocopherol for lipids, no significant changes in α-tocopherol/lipids were observed in either group. In the only other study to report both α-tocopherol and α-tocopherol/lipid concentrations (103), differential responses in the AO and PL supplemented groups were also explained by fluctuations in lipoproteins. Thus, the increase in plasma α-tocopherol concentrations during the exercise may be entirely the result of fluctuations in lipoprotein concentrations.

VITAMIN E AND LIPID PEROXIDATION

We observed faster d_6-*RRR*-α-tocopherol disappearance rates during the run, when lipid peroxidation levels were maximal, compared to the preceding rest day and the recovery period, consistent with our previous findings that endurance exercise increases disappearance of deuterium labeled α-tocopherol (1).

Plasma tocopherols are transported entirely within lipoproteins and fluctuate with lipoprotein concentrations (178). Due to this relationship between α-tocopherol concentrations and plasma lipids, we asked the question: Are the observed differences in d_6-α-tocopherol disappearance rates explained by fluctuations in lipid levels with exercise? Plasma lipids (total cholesterol + triglycerides) increased during the race, but decreased to below pre-race levels 2 h post-race and remained low up to 2 (cholesterol) and 3 (triglycerides) days post-race. Correction of d_6 α-tocopherol concentrations for fluctuations in cholesterol and triglycerides yielded somewhat different kinetics than d_6-α-tocopherol concentrations. Like d_6-α-tocopherol, disappearance of d_6-α-tocopherol /lipids was faster in the AO compared to the PL group at all

time periods and disappearance of d_6-α-tocopherol /lipids was faster in women compared with men. However, rates of d_6-α-tocopherol /lipids disappearance were similar during the pre-race and race periods for all 4 groups. These findings suggest that increased lipoprotein turnover accounts for the faster d_6-α-tocopherol disappearance observed during the race compared to pre-race.

Considering that the runners in the PL but not the AO group, experienced increased lipid peroxidation, it was an unexpected finding that d_6-α-tocopherol disappearance rates were faster in the AO compared with the PL group. One explanation is that metabolism of α-tocopherol by cytochrome p450s in the liver is up-regulated in response to vitamin E supplementation resulting in increased α-tocopherol disappearance independent of oxidative stress. Recent studies examining α-tocopherol binding to the nuclear receptor PXR (183) suggest that excess hepatic α-tocopherol may up-regulate its own metabolism (184). Thus, increased plasma d_6 disappearance in the AO groups may be explained in part by the 5-10 fold increases in non-oxidative metabolism of unlabeled α-tocopherol to α-CEHC in the AO group.

Faster d_6-α-tocopherol disappearance rates in women than in men were not explained by fluctuations in plasma lipid concentrations or increases in non-oxidative metabolism of α-tocopherol to α-CEHC. However, these findings suggest that vitamin E protected women more effectively in that they also had a faster decrease in F_2-IsoPs during recovery. Overall, our data suggest that women may have been more protected against oxidative stress due to an increased ability to utilize α-tocopherol. Furthermore, the higher HDL concentrations in women compared to men suggest that HDL delivery to the liver may be important in plasma vitamin E clearance.

MUSCLE DAMAGE

The ultramarathon run resulted in substantial increases in muscle damage markers and deficits in maximal force production of the knee extensors and flexors. Prior supplementation with vitamins E and C appeared to have no alleviating effect on muscle damage and/or fatigue. Alternatively, the ultramarathon run may have been so damaging as to mask or even overwhelm the protective effects of the antioxidants. For example, vitamin C was found to be protective in a more moderate exercise protocol, 60 minutes of box-stepping exercise, where supplementation enhanced the rate of recovery of MVC force deficit (187).

CK is a commonly used marker of muscle damage; it is well documented that CK increases in response to marathon running (61). In the present study, CK was elevated at post-race, but did not reach maximal concentrations until 24 h post-race consistent with previous reports (61). Our observation that combined supplementation with 300 mg vitamin E and 1000 mg vitamin C had no effect on exercise-related increases in CK is in agreement with the study of Petersen et al. (107). They reported no influence of 2 w supplementation with 400 mg vitamin E and 500 mg vitamin C on increases in CK following a 90 minute treadmill run (107). Our findings are in contrast, however, to those of Rokitzki et al. (13), who reported that 4.5 w supplementation with 400 IU vitamin E and 200 mg vitamin C attenuated increases in CK following a 90 km ultramarathon. Thus, results appear to be influenced not only by level of exercise, but also by the amount, duration and the type of supplement antioxidant.

LDH, another marker of muscle damage, also increases in response to marathon running (189). Vitamin E and C supplementation had no effect on

LDH increases following distance running. This finding differs from the report of Itoh et al. (129) that supplementation with 1200 IU/day α-tocopherol for 4 w attenuated LDH increases following 6 successive days of running, suggesting that a larger dose of vitamin E is required to attenuate muscle damage resulting from endurance exercise.

An alternative explanation for the lack of protection by antioxidants against muscle damage and inflammation is that the placebo group had relatively high plasma ascorbic acid levels (78 ± 9 μM). Levine et al. (99) has demonstrated that leukocyte ascorbic acid levels reach saturation with plasma levels of 50-60 μM. Saturation levels of ascorbic acid in leukocytes in both the antioxidant group and the placebo group could explain the lack of a treatment effect. For these reasons, Levine et al. (99) postulated that in order to test for a treatment effect of vitamin C, subjects in the placebo group would need to consume less than 100 mg of vitamin C per day from foods. Subjects in the present study were instructed to consume a prescribed diet consisting of foods low in vitamins C and E; vitamin C intake was limited to <50 mg during the race period. However, the high average daily caloric intake required to sustain the energy expenditure of training (2500-3000 kcal/day), likely led to consumption of ~100 mg of vitamin C per day despite the relatively small amounts of vitamin C in the low vitamin C foods subjects consumed.

GENDER DIFFERENCES IN RUNNERS

Men and women responded differently both to the antioxidant supplementation and to the exercise bout. Women and men in the present study were of similar fitness levels (VO_{2max}), but as is typical, men exhibited a higher proportion of lean body tissue (14), likely contributing to a higher metabolic rate (101). Thus, one explanation for the observed gender

differences is a higher metabolic rate in men leading to increased mitochondrial oxygen flux and increased production of ROS (87). Another possible explanatory variable, the female hormone estrogen, which exhibits antioxidant properties (87) and stabilizes cell membranes (190) and thus may have contributed to the faster decrease in F_2IsoPs observed in PL women.

HDL cholesterol is a known protective risk factor in cardiovascular disease, an oxidative stress disorder. Higher HDL concentrations are another possible source of protection in women. Following the race, elevated plasma F_2-IsoP concentrations in the PL women returned rapidly to pre-race concentrations, but remained elevated in the PL men (2). This difference between PL women and men may be explained by differences in α-tocopherol utilization (d_6 disappearance) as disappearance rates of both d_6 and d_6/lipids were faster in PL women during the race and during recovery. Greater vitamin E utilization and faster recovery from lipid peroxidation in women suggest that women may have been more protected against oxidative stress due to an increased ability to utilize vitamin E, but it does not explain why women had higher levels of DNA damage than men. These findings highlight the need for future studies investigating the effects of antioxidant supplementation on exercise-induced oxidative damage to compare both men and women.

CONCLUSIONS

Overall, as presented schematically in **Figure 18**, ROS generated in response to exercise can cause oxidative damage (1), stimulate an inflammatory response (106) and cause skeletal cell muscle damage (25, 43). Therefore, AO supplementation hypothetically could prevent exercise–induced oxidative stress, consequently protecting against oxidative damage,

inflammation and muscle damage (37, 43, 106). Unlike previous studies testing this hypothesis, the present study investigated the effects of supplementation with *both* vitamins E and C, by measuring oxidative stress, inflammatory *and* muscle damage markers in response to exercise. Supplementation *prevented* lipid peroxidation, but had no apparent effect on DNA oxidation, inflammation, muscle damage, or fatigue. These results suggest that the mechanism of oxidative damage is operating independently of the inflammatory and muscle damage responses (37). Alternatively, it may be that higher doses of α-tocopherol ($\geq$ 800 IU/day) are required to elicit an anti-inflammatory effect, as has been previously suggested (168, 172). It has been proposed that muscle fiber damage and necrosis stimulate an inflammatory response and subsequent repair/adaptation process (190). The present study provides some evidence that muscle damage and inflammation are linked. CK was significantly correlated with the acute phase protein CRP (2). If in fact, damaging exercise is required to elicit an adaptation response in muscle (169), prevention of muscle damage by antioxidants could blunt the training effect of exercise.

Preventing production/enhancing clearance of F_2-IsoPs may be more beneficial than preventing inflammation. F_2-IsoPs have demonstrated pro-atherogenic biological activity, including vasoconstriction, and activation of platelet aggregation (37, 76) and they are known to recruit pro-atherogenic monocytes and induce monocyte adhesion (173). In contrast, the muscle damage-induced inflammatory response stimulates recovery from exercise by inducing regeneration of damaged tissue and recruitment of satellite cell proliferation (146). Together, AO supplementation proved to prevent the damaging increase in lipid peroxidation without influencing inflammation. This

is especially important since prevention of exercise-induced inflammation could actually inhibit muscular adaptation to physical activity, the so-called "training effect" of exercise.

The acute oxidative and inflammatory stress responses characteristic of vigorous aerobic exercise are analogous to the stress responses observed following acute events such as myocardial infarction, ischemic stroke, surgery and trauma (25, 145, 174, 191, 192). Thus, aerobic exercise provides a well-controlled, relatively non-invasive model to study the effects of AO supplementation on acute oxidative and inflammatory stress. We are hopeful that results from this research may be useful for the exploration of these other chronic human diseases.

BIBLIOGRAPHY

1. Mastaloudis, A., Leonard, S., and Traber, M. (2001) Oxidative stress in athletes during extreme endurance exercise. *Free Radical Biology & Medicine* 31, 911-922

2. Mastaloudis, A., Morrow, J., Hopkins, D., Devaraj, S., and Traber, M. (2004) Antioxidant supplementation prevents exercise-induced lipid peroxidation, but not inflammation, in ultramarathon runners. *Free Radical Biology & Medicine*, 36(10), 1329-1341

3. Alessio, H. (2000) Lipid peroxidation in healthy and diseased models: influence of different types of exercise. In *Handbook of oxidants and antioxidants in exercise* (Sen, C., Packer, L., and Hanninen, O., eds) pp. 115-128, Elselvier, Amsterdam

4. Ashton, T., Rowlands, C., Jones, E., Young, I., Jackson, S., Davies, B., and Peters, J. (1998) Electron spin resonance spectroscopic detection of oxygen-centered radicals in human serum following exhaustive exercise. *European Journal of Applied Physiology* 77, 498-502

5. Child, R. B., Wilkinson, D. M., and Fallowfield, J. L. (2000) Effects of a training taper on tissue damage indices, serum antioxidant capacity and half-marathon running performance. *International Journal of Sport Medicine* 21, 325-331

6. Child, R. B., Wilkinson, D. M., Fallowfield, J. L., and Donnelly, A. E. (1998) Elevated serum antioxidant capacity and plasma malondialdehyde concentration in response to a simulated half-marathon run. *Medicine and Science in Sports and Exercise* 30, 1603-1607

7. Duthie, G. G., Robertson, J. D., Maughan, R. J., and Morrice, P. C. (1990) Blood antioxidant status and erythrocyte lipid peroxidation following distance running. *Archives of Biochemistry and Biophysics* 282, 78-83

8. Hessel, E., Haberland, A., Muller, M., Lerche, D., and Schimke, I. (2000) Oxygen radical generation of neutrophils: a reason for oxidative stress during marathon running? *Clinica Chimica Acta* 298, 145-156

9. Kanter, M., Lesmes, G., Kaminsky, L., La Ham-Saeger, J., and Nequin, N. (1988) Serum creatine kinase and lactate dehydrogenase changes following an eighty kilometer race. *European Journal of Applied Physiology* 57, 60-63

10. Lovlin, R., Cottle, W., Pyke, I., Kavanagh, M., and Belcastro, A. N. (1987) Are indices of free radical damage related to exercise intensity. *European Journal of Applied Physiology* 56, 313-316

11. Marzatico, F., Pansarasa, O., Bertorelli, L., Somenzini, L., and Della Valle, G. (1997) Blood free radical antioxidant enzymes and lipid

peroxides following long-distance and lactacidemic performances in highly trained aerobic and sprint trained athletes. *Journal of Sports Medicine and Physical Fitness* 37, 235-239

12. Maughan, R. J., Donnelly, A. E., Gleeson, M., Whiting, P. H., Walker, K. A., and Clough, P. J. (1989) Delayed-onset muscle damage and lipid peroxidation in man after a downhill run. *Muscle & Nerve* 12, 332-336

13. Rokitski, L., Logemann, E., Sagredos, A., Murphy, M., Wetzel-Roth, W., and Keul, J. (1994) Lipid peroxidation and antioxidative vitamins under extreme endurance stress. *Acta physiologica Scandinavica* 151, 149-158

14. Mastaloudis, A., Yu, D., Frei, B., Dashwood, R., and Traber, M. G. (2004) Endurance exercise results in DNA damage as detected by the comet assay. *Free Radical Biology & Medicine* 36(8), 966-975

15. Hartmann, A., Pfuhler, S., Dennog, C., Germadnik, D., Pilger, A., and Speit, G. (1998) Exercise-induced DNA effects in human leukocytes are not accompanied by increased formation of 8-hydroxy-2'-deoxyguanosine or induction of micronuclei. *Free Radical Biology & Medicine* 24, 245-251

16. Moller, P., Loft, S., Lundby, C., and Olsen, N. (2001) Acute hypoxia and hypoxic exercise induce DNA strand breaks and oxidative DNA damage in humans. *FASEB Journal* 15, 1181-1186

17. Niess, A., Baumann, M., Roecker, K., Horstmann, T., Mayer, F., and Dickhuth, H. H. (1998) Effects of intensive endurance exercise on DNA damage in leucocytes. *Journal of Sports Medicine and Physical Fitness* 38, 111-115

18. Okamura, K., Doi, T., Hamada, K., Sakurai, M., Yoshioka, Y., Mitsuzono, R., Migita, T., Sumida, S., and Sugawa-Katayama, Y. (1996) Effect of repeated exercise on urinary 8-hydroxydeoxyguanosine excretion in humans. *Free Radical Research* 26, 507-514

19. Tsai, K., Hsu, T.-G., Hsu, K.-M., Cheng, H., Liu, T.-Y., Hsu, C.-F., and Kong, C.-W. (2001) Oxidative DNA damage in human peripheral leukocytes induced by massive aerobic exercise. *Free Radical Biology & Medicine* 31, 1465-1472

20. Alessio, H. M., Hagerman, A., Fulkerson, B., Ambrose, J., Rice, R., and Wiley, R. (2000) Generation of reactive oxygen species after exhaustive aerobic and isometric exercise. *Medicine and Science in Sports and Exercise* 32, 1576-1581

21. Urso, M., and Clarkson, P. (2003) Oxidative stress, exercise, and antioxidant supplementation. *Toxicology* 189, 41-54

22. Tirosh, O., and Reznick, O. (2000) Chemical bases and biological relevance of protein oxidation. In *Handbook of Oxidants and Antioxidants in Exercise* (Sen, C. K., Packer, L., and Hanninen, O., eds) pp. 89-114, Elsevier, New York

23. Halliwell, B., and Gutteridge, J. M. C. (1999) *Free Radicals in Biology and Medicine*, Oxford University Press Inc., New York

24. Jackson, M. (2000) Exercise and oxygen radical production by muscle. In *Handbook of oxidants and antioxidants in exercise* (Sen, C., Packer, L., and Hanninen, O., eds) pp. 57-68, Elselvier, Amsterdam

25. Cannon, J. G., and Blumberg, J. B. (2000) Acute phase immune responses in exercise. In *Handbook of Oxidants and Antioxidants in Exercise* (Sen, C., Packer, L., and Hanninen, O., eds) pp. 177-194, Elsevier, New York

26. Traber, M. G. (1999) Vitamin E. In *Modern Nutrition in Health and Disease* (Shils, Olsen, Shike, and Ross, eds) pp. 347-362, Williams and Wilkens, Baltimore

27. Frei, B., England, L., and Ames, B. (1989) Ascorbate is an outstanding antioxidant inhuman blood plasma. *Proceedings of the National Academy of Sciences, USA* 86, 6377-6381

28. Frei, B., Stocker, R., England, L., and Ames, B. (1990) Ascorbate: the most effective antioxidant in human blood plasma. In *Antioxidants in Therapy and Preventive Medicine* pp. 155-163, Plenum Press, New York

29. Alessio, H., Goldfarb, A., and Cao, G. (1997) Exercise-induced oxidative stress before and after vitamin C supplementation. *International Journal of Sport Nutrition* 7, 1-9

30. Ashton, T., Young, I., Peters, J., Jones, E., Jackson, S., Davies, B., and Rowlands, C. (1999) Electron spin resonance spectroscopy, exercise and oxidative stress: an ascorbic acid intervention study. *Journal of Applied Physiology* 87, 2032-2036

31. Meydani, M., Evans, W., Handelman, G., Biddle, L., Fielding, R., Meydani, S., Burrill, J., Fiatarone, M., Blumber, J., and Cannon, J. (1993) Protective effect of vitamin E on exercise-induced oxidative damage in young and older adults. *American Journal of Physiology* 33, R992-998

32. Sacheck, J. M., Milbury, P., Cannon, J. G., Roubenoff, R., and Blumberg, J. B. (2003) Effect of vitamin E and eccentric exercise on selected biomarkers of oxidative stress in young and elderly men. *Free Radical Biology & Medicine* 34, 1575-1588

33. Sanchez-Quesada, J., Jorba, O., Payes, A., Otal, C., Serra-Grima, R., Gonzalez-Sastre, F., and Ordonez-Llanos, J. (1998) Ascorbic acid inhibits the increase in low-density lipoprotein (LDL) susceptibility to oxidation and the proportion of electronegative LDL induced by intense aerobic exercise. *Coronary Artery Disease* 9, 249-255

34. Schroder, H., Navarro, E., Tramullas, A., Mora, J., and Galiano, D. (2000) Nutrition antioxidant status and oxidative stress in professional basketball players: effects of a three compound antioxidative supplement. *Nutrition* 21, 146-150

35. Sumida, S., Tanaka, K., H., K., and Nakadomo, F. (1989) Exercise-induced lipid peroxidation and leakage of enzymes before and after vitamin E supplementation. *International Journal of Biochemistry* 21, 835-838

36. Kanter, M., Nolte, L., and Holloszy, J. (1993) Effects of an antioxidant vitamin mixture on lipid peroxidation at rest and postexercise. *Journal of Applied Physiology* 74, 965-969

37. Nieman, D. C., Henson, D. A., McAnulty, S. R., McAnulty, L., Swick, N. S., Utter, A. C., Vinci, D. M., Opiela, S. J., and Morrow, J. D. (2002) Influence of vitamin C supplementation on oxidative and immune changes after an ultramarathon. *Journal of Applied Physiology* 92, 1970-1977

38. Thompson, D., Williams, C., Kingsley, M., Nicholas, S., Lakomy, H., McArdle, F., and Jackson, M. (2001) Muscle soreness and damage parameters after prolonged intermittent shuttle-running following acute vitamin C supplementation. *Nutrition* 22, 68-75

39. Vasankari, T., Kujala, U., Sarna, S., and Ahotupa, M. (1998) Effects of ascorbic acid and carbohydrate ingestion on exercise induced oxidative stress. *Journal of Sports Medicine and Physical Fitness* 38, 281-285

40. Vasankari, T. J., Kujala, U. M., Vasankari, T. M., Vuorimaa, T., and Ahotupa, M. (1997) Increased serum and low-density-lipoprotein antioxidant potential after antioxidant supplementation in endurance athletes. *American Journal of Clinical Nutrition* 65, 1052-1056

41. Childs, A., Jacobs, C., Kaminski, T., Halliwell, B., and Leeuwenburgh, C. (2001) Supplementation with vitamin C and N-acetyl-cysteine increases oxidative stress in humans after an acute muscle injury induced by eccentric exercise. *Free Radical Biology & Medicine* 31, 745-753

42. Kanter, M. (1995) Free radicals and Exercise: Effects of nutritional antioxidant supplementation. *Exercise and Sport Science Review* 23, 375-397

43. Sjodin, B., Hellsten Westing, Y., and Apple, F. (1990) Biochemical mechanisms for oxygen free radical formation during exercise. *Sports Medicine* 10, 236-254

44. Fitts, R. (1994) Cellular Mechanisms of Muscle Fatigue. *Physiological Reviews* 74, 49-94

45. Powers, S., and Howley, E. (1999) *Exercise Physiology*, McGraw-Hill, New York

46. Ebbeling, C., and Clarkson, P. (1989) Exercise-induced muscle damage and adaptation. *Sports Medicine* 7, 207-234

47. Niess, A., Passek, F., Lorenz, I., Schneider, E., Dickhuth, H., Northoff, J., and Fehrenbach, E. (1999) Expression of the antioxidant stress protein heme oxygenase-1 (HO-1) in human leukocytes. *Free Radical Biology & Medicine* 26, 184-192

48. Viguie, C., Frei, B., Shigenaga, M., Ames, B., Packer, L., and Brooks, G. (1993) Antioxidant status and indexes of oxidative stress during consecutive days of exercise. *Journal of Applied Physiology* 75, 566-572

49. Liu, M., Bergholm, R., Makimmattila, S., Lahdenpera, S., Valkonen, M., Hilden, H., Yki-Jarvinen, H., and Taskinen, M. (1999) A marathon run increases the susceptibility of LDL to oxidation in vitro and modifies plasma antioxidants. *American Journal of Physiology* 273, E1083-1091

50. Briviba, K., and Sies, H. (1994) Non-Enzymatic Antioxidant Defense Systems. In *Natural Antioxidants in Human Health and Disease* (Frei, B., ed) pp. 107-128, Academic Press, Inc, San Diego

51. Morrow, J. D., Frei, B., Longmire, A., Gaziano, J., Lynch, S., Shyr, Y., Strauss, W., Oates, J., and Roberts, J. (1995) Increase in circulating products of lipid peroxidation (F_2-isoprostanes) in smokers. *New England Journal of Medicine* 332, 1198-1203

52. Fletcher, G., Balady, G., Blair, S., Blumenthal, J., Caspersen, C., Chaitman, B., Epstein, S., Froelicher, E., Froelicher, V., Pina, I., and Pollock, M. (1996) Statement on exercise: benefits and recommendations for physical activity programs for all Americans. *Circulation* 94, 857-862

53. Saxton, J., Donnelly, A., and Roper, H. (1994) Indices of free-radical-mediated damage following maximum voluntary eccentric and concentric muscular work. *European Journal of Applied Physiology* 68, 189-193

54. Groff, J., Gropper, S., and Hust, S. (1999) *Advanced Nutrition and Human Metabolism*, Wadsworth/Thomson Learning, Belmont

55. Cassarino, D. S., and Bennett, J. P. (1999) An evaluation of the role of mitochondria in neurodegenerative diseases: mitochondrial mutations and oxidative pathology, protective nuclear responses, and cell death in neurodegeneration. *Brain Research Reviews* 29, 1-25

56. Jenkins, R. R., and Goldfarb, A. (1993) Introduction: oxidant stress, aging and exercise. *Medicine and Science in Sports and Exercise* 25, 210-212

57. Jenkins, R., and Beard, J. (2000) Metal binding agents: possible role in exercise. In *Handbook of Oxidants and Antioxidants in Exercise* (Sen, C., Packer, L., and Hanninen, O., eds) pp. 129-152, Elsevier, Amsterdam

58. Kehrer, J., and Smith, C. (1994) Free radicals in biology: sources, reactivities, and roles in the etiology of human disease. In *Natural Antioxidants in Human Health and Disease* pp. 25-56, Academic Press, Inc, San Diego

59. Miller, B., Pate, R., and Burgess, W. (1988) Foot impact force and intravascular hemolysis during distance running. *International Journal of Sports Medicine* 9, 56-60

60. O'Toole, M., Hiller, W., Roalstad, M., and Douglas, P. (1987) Hemolysis during triathlon races: its relation to race distance. *Medicine and Science in Sports and Exercise* 20, 272-275

61. Armstrong, R. (1986) Muscle damage and endurance events. *Sports Medicine* 3, 370-381

62. Byrd, S. (1992) Alterations in the sarcoplasmic reticulum: a possible link to exercise-induced muscle damage. *Medicine and Science in Sports and Exercise* 24, 531-536

63. Morgan, D., and Allen, D. (1999) Early events in stretch-induced muscle damage. *Journal of Applied Physiology* 87, 2007-2015

64. Stewart, J., Ahlquist, D., McGill, D., Ilstrup, D., Schwartz, S., and Owen, R. (1984) Gastrointestinal blood loss and anemia in runners. *Annals of Internal Medicine* 100, 843-845

65. Ames, B., Cathcart, R., Schwiers, E., and Hochstein, P. (1981) Uric acid provides an antioxidant defense in humans against oxidant- and radical-caused aging and cancer; a hypothesis. *Proceedings of the National Academy of Sciences, USA* 78, 6858-6862

66. Pryor, W. (1994) Oxidants and Antioxidants. In *Natural Antioxidants in Human Health and Disease* (Frei, B., ed) pp. 1-24, Academic Press, Inc, San Diego

67. Alessio, H. M. (1993) Exercise-induced oxidative stress. *Medicine and Science in Sports and Exercise* 25, 218-224

68. Moore, K., and Roberts, J. (1998) Measurement of lipid peroxidation. *Free Radical Research* 28, 659-671

69. Asmus, K.-D., and Bonifacic, M. (2000) Introduction to free radicals. In *Handbook of oxidants and antioxidants in exercise* (Sen, C., Packer, L., and Hanninen, O., eds) pp. 3-56, Elsevier, Amsterdam

70. Halliwell, B., and Grootveld, M. (1987) The measurement of free radical reactions in humans. Some thoughts for future experimentation. *FEBS Letters* 213, 9-14

71. Han, D., Loukianoff, S., and McLaughlin, L. (2000) Oxidative stress indices: analytical aspects and significance. In *Handbook of Oxidants and Antioxidants in Exercise* (Sen, C., Packer, L., and Hanninen, O., eds) pp. 433-485, Elsevier, Amsterdam

72. Morrow, J., Hill, K., Burk, R., Nammour, T., Badr, K., and Roberts, J. (1990) A series of prostaglandin F_2-like compounds are produced *in vivo* in humans by a non-cyclooxygenase, free radical catalyzed mechanism. *Proceedings of the National Academy of Sciences, USA* 87, 9383-9387

73. Roberts, J. (1997) The generation and actions of isoprostanes. *Biochimica et Biophysica Acta* 1345, 121-135

74. Morrow, J., Chen, Y., Brame, C., Yang, J., Sanchez, S., Xu, J., Zackert, W., Awad, J., and Roberts, J. (1999) The isoprostanes: unique

prostaglandin-like products of free-radical-initiated lipid peroxidation. *Drug Metabolism Reviews* 31, 117-139

75. Morrow, J., and Roberts, L. (1997) The isoprostanes: unique bioactive products of lipid peroxidation. *Progress in Lipid Research* 36, 1-22

76. Roberts, J. I., and Morrow, J. D. (2000) Measurement of F_2-isoprostanes as an index of oxidative stress in vivo. *Free Radical Biology & Medicine* 28, 505-513

77. Pratico, D., Lee, V., Trojanowski, J., Rokach, J., and Fitzgerald, G. (1998) Increased F_2-isoprostanes in Alzheimer's Disease: evidence for enhanced lipid peroxidation in vivo. *FASEB Journal* 12, 1777-1783

78. Collins, C., Quaggiotto, P., Wood, L., O'Loughlin, E., Henry, R., and Garg, M. (1999) Elevated plasma levels of $F_{2\alpha}$-Isoprostane in Cystic Fibrosis. *Lipids* 34, 551-556

79. Wood, L., Fitzgerald, D., Gibson, P., Cooper, D., and Garg, M. (2000) Lipid peroxidation as determined by plasma isoprostanes is related to disease severity in mild asthma. *Lipids* 35, 967-974

80. Minuz, P., Andrioli, G., Degan, M., Gaino, S., Ortolani, R., Tommasoli, R., Zuliani, V., Lechi, A., and Lechi, C. (1998) The F_2-isoprostane 8-epiprostaglandin $F_2\alpha$ increases platelet adhesion and reduces the antiadhesive and antiaggregatory effects of NO. *Arteriosclerosis Thrombosis and Vascular Biology* 18, 1248-1256

81. O'Byrne, D., Devaraj, S., Grunert-Fuchs, M., and Jialal, I. (2002) Comparison of the antioxidant effects of Concord grape juice flavonoids and α-tocopherol on markers of oxidative stress in healthy adults. *American Journal of Clinical Nutrition* 76, 1367-1374

82. Simons, L., von Konigsmark, M., Simons, J., Stocker, R., and Celermajer, D. (1999) Vitamin E ingestion does not improve arterial endothelial dysfunction in older adults. *Atherosclerosis* 143, 193-199

83. Meagher, E., Barry, O., Lawson, J., Rokach, J., and Fitzerald, G. (2001) Effects of vitamin E on lipid peroxidation in healthy persons. *Journal of the American Medical Association* 285, 1178-1182

84. Marangon, K., Devaraj, S., Tirosh, O., Packer, L., and Jialal, I. (1999) Comparison of the effect of α-lipoic acid and α-tocopherol supplementation on measures of oxidative stress. *Free Radical Biology & Medicine* 27, 1114-1121

85. Upritchard, J., Schuurman, C., Wiersma, A., Tijburg, L., Coolen, S., Rijken, J., and Wiseman, S. (2003) Spread supplemented with moderate doses of vitamin E and carotenoids reduces lipid peroxidation in healthy, non-smoking adults. *American Journal of Clinical Nutrition* 78, 985-992

86. Huang, H., Appel, L., Croft, K., Miller, E., Mori, T., and Puddey, I. (2002) Effects of vitamin C and vitamin E on in vivo lipid peroxidation: results

of a randomized controlled trial. *American Journal of Clinical Nutrition* 76, 549-555

87. Ide, T., Tsutsui, H., Ohashi, N., Hayashidani, S., Suimatsu, N., Tsuchihashi, M., Tamai, H., and Takeshita, A. (2002) Greater oxidative stress in healthy young men compared with premenopausal women. *Arteriosclerosis Thrombosis and Vascular Biology* 22, 438-442

88. Reilly, M., Delanty, N., Lawson, J., and Fitzerald, G. (1996) Modulation of oxidant stress in vivo in chronic cigarette smokers. *Circulation* 94, 19-25

89. Dietrich, M., Block, G., Hudes, M., Morrow, J. D., Norkus, E., Traber, M. G., Cross, C., and Packer, L. (2002) Antioxidant supplementation decreases lipid peroxidation biomarker F_2-isoprostanes in plasma of smokers. *Cancer Epidemiology, Biomarkers & Prevention* 11, 7-13

90. Darko, D., Dornhorst, A., Kelly, F., Ritter, J., and Chowienczyk, P. (2002) Lack of effect of oral vitamin C on blood pressure, oxidative stress and endothelial function in Type II diabetes. *Clinical Science* 103, 339-344

91. Davi, G., Ciabattoni, G., Consoli, A., Mezzetti, A., Falco, A., Santarone, S., Pennese, E., Vitacolonna, E., Bucciarelli, T., Costantini, F., Capani, F., and Patrono, C. (1999) In vivo formation of 8-iso-protaglandin $F_{2\alpha}$ and platelet activation in diabetes mellitus. *Circulation* 99, 224-229

92. Brigelius-Flohe, R., and Traber, M. G. (1999) Vitamin E: function and metabolism. *FASEB Journal* 13, 1145-1155

93. Burton, G., Traber, M., and Acuff, R. (1998) Human plasma and tissue α-tocopherol concentrations in response to supplementation with deuterated natural and synthetic vitamin E. *American Journal of Clinical Nutrition* 67, 669-684

94. Institute of Medicine, Food and Nutrition Board (2000) *Dietary reference intakes for vitamin C, vitamin E, Selenium and carotenoids*, National Academy Press, Washington D.C

95. Meydani, M. (1995) Vitamin E. *The Lancet* 345, 170-175

96. Traber, M., Ramakrishnan, R., and Kayden, H. (1994) Human plasma vitamin E kinetics demonstrate rapid recycling of plasma RRR-α-tocopherol. *Proceedings of the National Academy of the Sciences, USA* 91, 10005-10008

97. Carr, A., and Frei, B. (1999) Does vitamin C act as a pro-oxidant under physiological conditions? *FASEB Journal* 13, 1007-1024

98. Tamai, H., Kim, H., Hozumi, M., Kuno, T., Murata, T., and Morinobu, T. (2000) Plasma α-tocopherol level in diabetes mellitus. *BioFactors* 11, 7-9

99. Levine, M., Conry-Cantilena, C., Wang, Y., Welch, R., Washko, P., Dhariwal, K., Park, J., Lazarev, A., Graumlich, J., King, J., and Cantilena, L. (1996) Vitamin C pharmocokinetics in healthy volunteers:

Evidence for a recommended dietary allowance. *Proceedings of the National Academy of Sciences, USA* 93, 3704-3709

100. Hellsten, Y., Tullson, P., Richter, E., and Bangsbo, J. (1997) Oxidation of urate in human skeletal muscle during exercise. *Free Radical Biology & Medicine* 22, 169-174

101. Brooks, G., Fahey, T., White, T., and Baldwin, K. (2000) *Exercise Physiology Human Bioenergetics and Its Applications*, Mayfield Publishing Company, Mountain View

102. Hellsten, Y. (2000) The role of xanthine oxidase in exercise. In *Handbook of Oxidants and Antioxidants in Exercise* (Sen, C., Packer, L., and Hanninen, O., eds) pp. 153-176, Elsevier, Amsterdam

103. Buchman, A., Killip, D., Ou, C., Rognerud, C., Pownall, H., Dennis, K., and Dunn, J. (1998) Short-term vitamin E supplementation before marathon running: a placebo-controlled trial. *Nutrition* 15, 278-283

104. Cannon, J., Meydani, S., Fielding, R., Fiatarone, M., Meydani, M., Farhangmehr, M., Orencole, S., Blumberg, J., and Evans, W. (1991) Acute phase response in exercise. II. Associations between vitamin E, cytokines, and muscle proteolysis. *American Journal of Physiology* 260, R1235-R1240

105. Singh, A., Papanicolaou, D. A., Lawrence, L. L., Howell, E. A., Chrousos, G. P., and Deuster, P. A. (1999) Neuroendocrine responses to running in women after zinc and vitamin E supplementation. *Medicine and Science in Sports and Exercise* 31, 536-542

106. Vassilakopoulos, T., Karatza, M., Katsaounou, P., Kollintza, A., Zakynthinos, S., and Roussos, C. (2003) Antioxidants attenuate the plasma cytokine response to exercise in humans. *Journal of Applied Physiology* 94, 1025-1032

107. Petersen, E., Ostrowski, K., Ibfelt, T., Richelle, M., Offord, E., Halkjaer-Kristensen, J., and Pedersen, B. K. (2001) Effect of vitamin supplementation on cytokine response and on muscle damage after strenuous exercise. *American Journal of Physiology* 280, C1570-C1575

108. Pfeiffer, J., Askew, E., Roberts, D., Wood, S., Benson, J., Johnson, S., and Freedman, M. (1999) Effect of antioxidant supplementation on urine and blood markers of oxidative stress during extended moderate-altitude training. *Wilderness and Environmental Medicine* 10, 66-74

109. Balakrishnan, S. D., and Anuradha, C. D. (1998) Exercise, depletion of antioxidants and antioxidant manipulation. *Cell Biochemistry and Function* 16, 269-275

110. Kaikkonen, J., Kosonen, L., Nyyssonen, K., Porkkala-Sarataho, E., Salonen, R., Dorpela, H., and Salonen, J. (1998) Effect of combined coenzyme Q10 and d-α-tocopheryl acetate supplementation on exercise-induced lipid peroxidation and muscular damage: a placebo-controlled double-blind study in marathon runners. *Free Radical Research* 29, 85-92

111. Peters, E., Anderson, R., and Theron, A. (2001) Attenuation of increase in circulating cortisol and enhancement of the acute phase protein response in vitamin C-supplemented ultramarathoners. *International Journal of Sport Medicine* 22, 120-126

112. Peters, E., Anderson, R., Nieman, D., Fickl, H., and Iogessa, V. (2001) Vitamin C supplementation attenuates the increases in circulating cortisol, adrenaline, and anti-inflammatory polypeptides following ultramarathon running. *International Journal of Sport Medicine* 22, 537-543

113. Koz, M., Erbas, D., Bilgihan, A., and Aricioglu, A. (1992) Effects of acute swimming exercise on muscle and erythrocyte malondialdehyde, serum myoglobin and plasma ascorbic acid concentrations. *Canadian Journal of Physiological Pharmacology* 70, 1392-1395

114. Hornsby, P., and Crivello, J. (1983) The role of lipid peroxidation and biological antioxidants in the function of the adrenal cortex. Part 1. A background review. *Molecular and Cellular Endocrinology* 30, 1-20

115. Gleeson, M., Robertson, J., and Maughan, R. (1987) Influence of exercise on ascorbic acid status in man. *Clinical Science* 73, 501-505

116. Nieman, D., Peters, E., Henson, D., Nevines, E., and Thompson, M. (2000) Influence of vitamin C supplementation on cytokine changes following an ultramarathon. *Journal of Interferon and Cytokine Research* 20, 1029-1030

117. Goodman, C., Henry, G., Sawson, B., Gillam, I., Beilby, J., Ching, S., Fabian, V., Dasig, D., Kakulas, B., and Morling, P. (1997.) Biochemistry and ultrastructural indices of muscle damage after a 21-km run. *The Australian Journal of Science and Medicine in Sport* 29, 95-98

118. Pyne, D. (1994) Exercise induced muscle damage and inflammation: a review. *The Australian Journal of Science and Medicine in Sport* 26, 49-58

119. Inayama, T., Kumagai, Y., Sakane, M., Saito, M., and Matsuda, M. (1996) Plasma protein-bound sulfhydryl group oxidation in humans following a full marathon race. *Life Sciences* 59, 573-578

120. Stein, T., and Leskiw, M. (2000) Oxidant damage during and after spaceflight. *American Journal of Physiology* 278, E375-382

121. Mori, T., Dunstan, D., Burke, V., Croft, K., Rivera, J., Beilin, L., and Puddey, I. (1999) Effect of dietary fish and exercise training on urinary F_2-isoprostane excretion in non-insulin-dependent diabetic patients. *Metabolism* 48, 1402-1408

122. Case, D., Baer, J. T., and Subbiah, M. T. R. (1999) The effect of prolonged exercise on lipid peroxidation in eumenorrheic female runners. *Medicine and Science in Sports and Exercise* 31, 1390-1393

123. Shern-Brewer, R., Santanam, N., Wetzstein, C., White-Welkley, J., Price, L., and Parthasarathy, S. (2000) The paradoxical relationship of aerobic exercise and the oxidative theory of atherosclerosis. In

Handbook of oxidants and antioxidants in exercise (Sen, C., Packer, L., and Hanninen, O., eds) pp. 1053-1069, Elsevier, Amsterdam

124. Koot, R., Amelink, G., Blankenstein, M., and Bar, P. (1991) Tamoxifen and oestrogen both protect the rat muscle against physiological damage. *Journal of Steroid Biochemistry and Molecular Biology* 40, 689-695

125. Mastaloudis, A., Widrick, J., Berry, C., Leonard, S., and Traber, M. (2001) An exhaustive treadmill run does not cause oxidative stress in moderately trained female runners. *FASEB Journal* 15, A951

126. Wetzstein, C., Shern-Brewer, R., Santanam, N., Green, N., White-Welkley, J., and Parthasarathy, S. (1998) Does acute exercise affect the susceptibility of low density lipoprotein to oxidation? *Free Radical Biology & Medicine* 24, 679-682

127. Shern-Brewer, R., Santanam, N., Wetzstein, C., White-Welkley, J., and Parthasarathy, S. (1998) Exercise and cardiovascular disease, a new perspective. *Arteriosclerosis Thrombosis and Vascular Biology* 18, 1181-1187

128. Oostenbrug, G., Mensink, R., Hardeman, M., De Vries, T., Brouns, F., and Hornstra, G. (1997) Exercise performance, red blood cell deformability, and lipid peroxidation: effects of fish oil and vitamin E. *Journal of Applied Physiology* 83, 746-752

129. Itoh, H., Ohkuwa, T., Yamazaki, Y., Shimoda, T., Wakayama, A., Tamura, S., Yamamoto, T., Sato, Y., and Miyamura, M. (2000) Vitamin E supplementation attenuates leakage of enzymes following 6 successive days of running training. *International Journal of Sports Medicine* 21, 369-374

130. Devaraj, S., Adams-Huet, B., Fuller, C., and Jialal, I. (1997) Dose-response comparison of RRR-alpha-tocopherol and all-racemic alpha-tocopherol on LDL oxidation. *Arteriosclerosis Thrombosis and Vascular Biology* 17, 2273-2279

131. Fuller, C., Grundy, S., Norkus, E., and Jialal, I. (1996) Effect of ascorbate supplementation on low density lipoprotein oxidation in smokers. *Atherosclerosis* 119, 139-150

132. Hartmann, A., and Niess, A. (2000) Oxidative DNA damage in exercise. In *Handbook of Oxidants and Antioxidants in Exercise* (Sen, C., Packer, L., and Hanninen, O., eds) pp. 195-217, Elsevier, Amsterdam

133. Inoue, T., Mu, Z., Sumikawa, K., Adachi, K., and Okochi, T. (1993) Effect of physical exercise on the content of 8-hydroxydeoxyguanosine in nuclear DNA prepared from human lymphocytes. *Japanese Journal of Cancer Research* 84, 720-725

134. Poulsen, H., Loft, S., and Vistisen, K. (1996) Extreme exercise and oxidative DNA modification. *Journal of Sports Sciences* 14, 343-346

135. Loft, S., Astrup, A., Buemann, B., and Poulsen, H. (1994) Oxidative DNA damage correlates with oxygen consumption in humans. *FASEB Journal* 8, 534-537

136. Collins, A. (1998) The comet assay: a novel approach to measuring DNA oxidation. In *DNA & Free Radicals: Techniques, Mechanisms & Applications* (Aruoma, O., and Halliwell, B., eds) pp. 241-260, OICA International, London

137. Speit, G., Dennog, C., and Lampl, L. (1998) Biological significance of DNA damage induced by hyperbaric oxygen. *Mutagenesis* 13, 85-87

138. Hartmann, A., Niess, A., Grunert-Fuchs, M., Poch, B., and Speit, G. (1995) Vitamin E prevents exercise-induced DNA damage. *Mutation Research* 346, 195-202

139. Mars, M., Governder, S., Weston, A., Naicker, V., and Chuturgoon, A. (1998) High intensity exercise: a cause of lymphocyte apoptosis? *Biochemical and Biophysical Research Communications* 249, 366-370

140. Niess, A., Hartmann, A., Grunert-Fuchs, M., Poch, B., and Speit, G. (1996) DNA damage after exhaustive treadmill running in trained and untrained men. *International Journal of Sport Medicine* 17, 397-403

141. Hartmann, A., Plappert, U., Raddatz, K., Grunert-Fuchs, M., and Speit, G. (1994) Does physical activity induce DNA damage? *Mutagenesis* 9, 269-272

142. Moller, P., and Loft, S. (2002) Oxidative DNA damage in human white blood cells in dietary antioxidant intervention studies. *American Journal of Clinical Nutrition* 76, 303-310

143. Pedersen, B. K., Ostrowski, K., Rohde, T., and Bruunsgaard, H. (1998) The cytokine response to strenuous exercise. *Canadian Journal of Physiological Pharmacology* 76, 505-511

144. Pedersen, B. K., Bruunsgaard, H., Ostrowski, K., Krabbe, K., Hansen, H., Krzywkowski, K., Toft, A., Sondergaard, S. R., Petersen, E. W., Ibfelt, T., and Schjerling, P. (2000) Cytokines in aging and exercise. *International Journal of Sport Medicine* 21 Suppl 1, S4-9

145. Nieman, D. C. (1997) Immune response to heavy exertion. *Journal of Applied Physiology* 82, 1385-1394

146. Malm, C. (2001) Exercise-induced muscle damage and inflammation: fact or fiction? *Acta Physiologica Scandinavica* 171, 233-239

147. Cannon, J., Orencole, S., Fielding, R., Meydani, M., Meydani, S., Fiatarone, M., Blumberg, J., and Evans, W. (1990) Acute phase response in exercise: interaction of age and vitamin E on neutrophils and muscle enzyme release. *American Journal of Physiology* 259, R1214-R1219

148. Kuehl, R. (2000) *Design of Experiments: Statistical Principles of Research Design and Analysis*, Duxbury Press, Pacific Grove

149. Anderson, D., Yu, T.-W., Wright, J., and Ioannides, C. (1998) An examination of DNA strand breakage in the comet assay and antioxidant capacity in diabetic patients. *Mutation Research* 398, 151-161

150. Banath, J., Kim, A., and Olive, P. (2001) Overnight lysis improves the efficiency of detection of DNA damage in the alkaline Comet assay. *Radiation Research* 155, 564-571

151. Tice, R. (1995) The single cell gel/Comet assay: a microgel electrophoretic technique for the detection of DNA damage and repair in individual cells. In *Environmental Mutagenesis* (Phillips, D. H., and Venitt, S., eds) pp. 315-339, BIOS Scientific Publishers Ltd, Oxford

152. Podda, M., Weber, C., Traber, M., and Packer, L. (1996) Simultaneous determination of tissue tocopherols, tocotrienols, ubiquinols, and ubiquinones. *Journal of Lipid Research* 37, 893-901

153. Kutnink, M., Hawkes, W., Schaus, E., and Omaye, S. (1987) An internal standard method for the unattended high-performance liquid chromatographic analysis of ascorbic acid in blood components. *Analytical Biochemistry* 166, 424-430

154. Der, G., and Everitt, B. (2002) *A Handbook of Statistical Analyses Using SAS*, Chapman and Hall/CRC, New York

155. Willy, C., Dahouk, S., Starck, C., Kaffenberger, W., Gerngrob, H., and Plappert, U. (2000) DNA damage in human leukocytes after ischemia/reperfusion injury. *Free Radical Biology & Medicine* 28, 1-12

156. Astley, S., Langrish-Smith, A., Southon, S., and Sampson, M. (1999) Vitamin E supplementation and oxidative damage to DNA and plasma LDL in Type 1 diabetes. *Diabetes Care* 22, 1626-1631

157. Sul, D., Oh, E., Im, H., Yang, M., Kim, C., and Lee, E. (2003) DNA damage in T- and B-lymphocytes and granulocytes in emission inspection and incineration workers exposed to polycyclic aromatic hydrocarbons. *Mutation Research* 538, 109-119

158. Ho, E., Courtemanche, C., and Ames, B. (2003) Zinc deficiency induces oxidative DNA damage and increases p53 expression in human lung fibroblasts. *Journal of Nutrition* 133, 2543-2548

159. Ho, E., and Ames, B. (2002) Low intracellular zinc induces oxidative DNA damage, disrupts p53, NFkappa B, and AP1 DNA binding, and affects DNA repair in a rat glioma cell line. *Proceedings of the National Academy of Sciences, USA* 99, 16770-16775

160. Collins, A., Olmedilla, B., Southon, S., Granado, F., and Duthie, S. (1998) Serum carotenoids and oxidative DNA damage in human lymphocytes. *Carcinogenesis* 19, 2159-2162

161. Bajpayee, M., Dhawan, A., Parmar, D., Pandey, A., Mathur, N., and Seth, P. (2002) Gender-related differences in basal DNA damage in lymphocytes of a healthy Indian population using the alkaline Comet assay. *Mutation Research* 520, 83-91

162. Kaiser, J. (2003) Sipping from a poisoned chalice. *Science* 302, 376-379

163. Thune, I., and Furberg, A. (2001) Physical activity and cancer risk: dose-response and cancer, all sites and site-specific. *Medicine and Science in Sports and Exercise* 33, S530-550

164. Astrand, P.-O., and Rodahl, K. (1986) *Textbook of work physiology: physiological basis of exercise*, McGraw Hill Book Company, New York

165. Mahler, D., Froelicher, V., Miller, N., and York, T. (1995) *ACSM's Guidelines for Exercise Testing and Prescription*, Williams & Wilkins, Philadelphia

166. Morrow, J., and Roberts, J. I. (1998) Mass spectrometric quantification of F_2-isoprostanes in biological fluids and tissues as a measure of oxidant stress. *Methods in Enzymology* 300, 3-12

167. Devaraj, S., and Jialal, I. (2000) Low-density lipoprotein postsecretory modification, monocyte function, and circulating adhesion molecules in type 2 diabetic patients with and without macrovascular complications: the effect of α-tocopherol supplementation. *Circulation* 102, 191 - 196

168. Devaraj, S., and Jialal, I. (2000) Alpha tocopherol supplementation decreases serum C-reactive protein and monocyte interleukin-6 levels in normal volunteers and type 2 diabetic patients. *Free Radical Biology & Medicine* 29, 790-792

169. Radak, Z., Taylor, A. W., Ohno, H., and Goto, S. (2001) Adaptation to exercise-induced oxidative stress: from muscle to brain. *Exercise and Immunological Reviews* 7, 90-107

170. Brull, D., Serrano, N., Zito, F., Jones, L., Montgomery, H., Rumley, A., Sharma, P., Lowe, G., World, M., Humphries, S., and Hingorani, A. (2004) Human CRP gene polymorphism influences CRP levels: implications for the prediction and pathogenesis of coronary heart disease. *Arteriosclerosis Thrombosis and Vascular Biology* 23, 2063-2069

171. Nieman, D. C., Davis, J., Henson, D. A., Walberg-Rankin, J., Shute, M., Dumke, C., Utter, A., Vinci, D. M., Carson, J., Brown, A., Lee, W., McAnulty, S. R., and McAnulty, L. (2003) Carbohydrate ingestion influences skeletal muscle cytokine mRNA and plasma cytokine levels after a 3-h run. *Journal of Applied Physiology* 94, 1917-1925

172. Jialal, I., and Devaraj, S. (2003) Antioxidants and atherosclerosis: don't throw the baby out with the bath water. *Circulation* 107, 926-928

173. Leitinger, N., Huber, J., Rizza, C., Mechtcheriakova, D., Bochkov, V., Koshelnick, Y., Berliner, J., and Binder, B. (2001) The isoprostane 8-iso-$PGF_{2\alpha}$ stimulates endothelial cells to bind monocytes: differences from thromboxane-mediated endothelial activation. *FASEB Journal* 15, 1254-1256

174. Fallon, K. E. (2001) The acute phase response and exercise: the ultramarathon as prototype exercise. *Clinical Journal of Sports Medicine* 11, 38-43

175. Lodge, J., Traber, M., Elsner, A., and Brigelius-Flohe, R. (2000) A rapid method for the extraction and determination of vitamin E metabolites in human urine. *Journal of Lipid Research* 41, 148-154

176. Himmelfarb, J., Kane, J., McMonagle, E., Zaltas, E., Bobzin, S., Boddupalli, S., Phinney, S., and Miller, G. (2003) Alpha and gamma tocopherol metabolism in healthy subjects and patients with end-stage renal disease. *Kidney International* 64, 978-991

177. Vaule, H., Leonard, S., and Traber, M. (2004) Vitamin E delivery to human skin; studies using deuterated α-tocopherol measured by APCI LC-MS. *Free Radical Biology & Medicine* 36, 456-463

178. Traber, M., and Jialal, I. (2000) Measurement of lipid-soluble vitamins-further adjustment needed? *Lancet* 355, 2013-2014

179. Kolleck, I., Schlame, M., Fechner, H., Looman, A., Wissel, H., and Rustow, B. (1999) HDL is the major source of vitamin E for type II pneumocytes. *Free Radical Biology & Medicine* 27, 882-890

180. Acton, S., Scherer, P., Lodish, H., and Krieger, M. (1994) Expression cloning of SR-BI, a CD36-related class B scavenger receptor. *Journal of Biological Chemistry* 269, 21003-21009

181. Devaraj, S., Hugou, I., and Jialal, I. (2001) Alpha-tocopherol decreases CD36 expression in human monocyte-derived macrophages. *Journal of Lipid Research* 42, 521-527

182. Ricciarelli, R., Zingg, J., and Azzi, A. (2000) Vitamin E reduces the uptake of oxidized LDL by inhibiting CD36 scavenger receptor expression in cultured aortic smooth muscle cells. *Circulation* 102, 82-87

183. Landes, N., Pfluger, P., Kluth, D., Birringer, M., Rühl, R., Böl, G., Glatt, H., and Brigelius-Flohé, R. (2003) Vitamin E activates gene expression via the pregnane X receptor. *Biochemical Pharmacology* 65, 269-273

184. Traber, M. (2004) Vitamin E, nuclear receptors and xenobiotic metabolism. *Archives of Biochemistry and Biophysics* 423, 6-11

185. Maxwell, S., Jakeman, P., Thomason, J., Leguen, C., and Thorpe, G. (1993) Changes in plasma antioxidant status during eccentric exercise and the effect of vitamin supplementation. *Free Radical Research* 19, 191-202

186. Millet, G. Y., Martin, V., Lattier, G., and Ballay, Y. (2003) Mechanisms contributing to knee extensor strength loss after prolonged running exercise. *Journal of Applied Physiology* 94, 193-198

187. Jakeman, P., and Maxwell, S. (1993) Effect of antioxidant vitamin supplementation on muscle function after eccentric exercise. *European Journal of Applied Physiology* 67, 426-430

188. Beaton, L. J., Allan, D. A., Tarnopolsky, M. A., Tiidus, P. M., and Phillips, S. M. (2002) Contraction-induced muscle damage is unaffected by vitamin E supplementation. *Medicine and Science in Sports and Exercise* 34, 798-805

189. Kanter, M., Kaminsky, L., La Ham-Saeger, J., Lesmes, G., and Nequin, N. (1986) Serum enzyme levels and lipid peroxidation in ultramarathon runners. *Annals of Sports Medicine* 3, 39-41

190. Stupka, N., Lowther, S., Chorneyko, K., Bourgeois, J., Hogben, C., and Tarnopolsky, M. (2000) Gender differences in muscle inflammation after eccentric exercise. *Journal of Applied Physiology* 89, 2325-2332

191. Heinrich, P. C., Castell, J. V., and Andus, T. (1990) Interleukin-6 and the acute phase response. *Biochemistry Journal* 265, 621-636

192. Pedersen, B. K., and Hoffman-Goetz, L. (2000) Exercise and the immune system: regulation, integration, and adaptation. *Physiological Reviews* 80, 1055-1081