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Lipid Peroxidation and Coupled Vitamin Oxidation in Simulated and Human Gastric Fluid Inhibited by Dietary Polyphenols: Health Implications

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The Western diet contains large quantities of oxidized lipids, because a large proportion of the food in the diet is consumed in a fried, heated, processed, or stored form. We investigated the reaction that could occur in the acidic pH of the stomach and accelerate the generation of lipid hydroperoxides and cooxidation of dietary vitamins. To estimate the oxygen content in the stomach after food consumption, oxygen released from masticated bread (20 g) into deoxygenated water (100 mL) was measured. Under these conditions, the oxygen concentration rose by 250 μM and reached a full oxygen saturation. The present study demonstrated that heated red meat homogenized in human gastric fluid, at pH 3.0, generated hydroperoxides and malondialdehyde. The cross-reaction between free radicals produced during this reaction cooxidized vitamin E, β-carotene, and vitamin C. Both lipid peroxidation and cooxidation of vitamin E and β-carotene were inhibited at pH 3.0 by red wine polyphenols. Ascorbic acid (44 mg) at a concentration that represented the amount that could be ingested during a meal inhibited lipid peroxidation only slightly. Red wine polyphenols failed to prevent ascorbic acid oxidation significantly but, in conjunction with ascorbic acid, did inhibit lipid peroxidation. In the presence of catechin, a well-known polyphenol found in red wine, ascorbic acid at pH 3.0 works in a synergistic manner preventing lipid peroxidation and β-carotene cooxidation. The present data may explain the major benefits to our health and the crucial role of consuming food products rich in dietary antioxidants such as fruits, vegetables, red wines, or green tea during the meal.

KEYWORDS: Lipid peroxidation; vitamins E and C; β-carotene; human gastric fluid; dietary polyphenols antioxidants; health

INTRODUCTION

Over the past 20 years, a large number of epidemiological studies have been conducted to investigate the relationships between the diet and the risks of developing cardiovascular diseases and various types of cancer (*1*−*5*). Epidemiological studies and experimental data suggest that diets high in fat and red meat are risk factors contributing to the development of atherogenesis and of several kinds of cancer, especially colon cancer (*6*−*12*).

The Western diet contains large quantities of oxidized fatty acids (*13*−*18*), oxidized cholesterol (*15*, *17*), and cytotoxic aldehydes and phospholipids (*19*−*24*), because a large proportion of the food in the diet is often consumed in a fried, heated, or processed form.

The cross-reactions between lipid free radicals, generated during lipid peroxidation, and other food constituents can dramatically alter the range of cytotoxic, mutagenic, and atherogenic compounds produced; these include hydroperoxides, oxycholesterol, malondialdehyde, and hydroxy alkanals (*13*−*24*). Repeated consumption of oxidized fat in the diet may pose a chronic threat to human health (*16*−*18*, *22*, *24*). Atherosclerosis may result from processes that occur following food consumption and that involve oxidized lipids in chylomicrons. High fat and high cholesterol foods not only affect endogenous lipoprotein production and catabolism but probably also lead to transient exposure of arteries to cytotoxic chylomicron remnants and lipolysis products (*25*−*28*). Most recently, we demonstrated that the stomach and its gastric fluid could be a site and medium for further dietary lipid peroxidation or antioxidation (*29*).

The aim of the present study was not only to demonstrate that cytotoxic compounds are generated during lipid peroxidation in simulated stomach medium but also to show that very

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important dietary vitamins such as vitamin E, β-carotene (provitamin A), and vitamin C are destroyed as a result. The generation of oxidation byproducts together with destruction of important dietary vitamins in general in the stomach are processes that may exacerbate the risk factors that affect our health.

MATERIALS AND METHODS

Metmyoglobin (metMb, from horse skeletal muscle), soybean lipoxygenase (type I-B), β-carotene, linoleic acid, Tween 20, butylated hydroxytoluene, catechin, pepsin (A, from porcine stomach mucosa), ferrous ammonium sulfate, xylenol orange, and triphenylphosphine (TPP) were obtained from Sigma Chemical Co. (St. Louis, MO). Sodium chloride, hydrogen peroxide (30%), and L-(+)-ascorbic acid (AA) were obtained from Merck (Darmstadt, Germany). Ferric chloride (Fe) was obtained from Riedel-de-Haen (Hannover, Germany). Sodium borohydride was from BDH (Poole, Dorset, England). Solvents were all high-performance liquid chromatography (HPLC) grade (J. T. Baker, Phillipsburg, NJ). Soybean oil, red wine (Israeli Cabernet Sauvignon), and grilled turkey meat (shawarma) were bought at commercial stores in Israel. Human gastric fluid (HGF) was collected with permission from fasting healthy volunteers during regular gastric endoscopic tests and kept at −80 °C pending use. Simulated gastric fluid (SGF) was freshly prepared according to the U.S. Pharmacopoeia (*30*). SGF contained 0.2% NaCl, 0.32% pepsin, and HCl to pH 2 or other pH values.

Hydroperoxides Measurement in Linoleic Acid Emulsions. Spectroscopic Measurement. Hydroperoxides were determined by means of the ferrous ion oxidation−xylenol orange (FOX2) method (*31*), including spectral analysis at 560 nm by comparison with an H_2O_2 standard curve. The reaction mixtures contained 2.7 mM linoleic acid, 0.14% (v/v) Tween 20, and 0.14% (v/v) ethanol as emulsifiers, in various solutions: 0.01 M acetate buffer (pH 7.0−3.0), HCl solutions (pH 3.0−1.5), SGF, and HGF. The catalysts (metMb, Fe-AA, or metMb-Fe-AA) and the antioxidants (red wine or catechin) were added to the reaction mixtures, as indicated in the Results section. Catechin was dissolved in 10% ethanol in water, so that the ethanol concentration in the reactions was 2%. The reaction tubes were incubated in triplicate in a shaking bath at 37 °C for 180 min. Samples of 25 or 50 μL were taken from the tubes during the incubation and added to 475 or 950 μL, respectively, of FOX2 reagent.

It had been confirmed in preliminary experiments that the presence of metMb, Fe, AA, wine, or catechin themselves, at the concentrations used, did not interfere with the FOX2 assay. Under the experimental conditions, the optical absorbance of the red wine pigments at 560 nm was up to 0.04 O.D.; therefore, the absorbance of control treatments in acidic methanol solution (as in the FOX2 reagent but without the xylenol orange) was deducted from the absorbance of the parallel experimental treatments.

Hydroperoxides Measurement in Turkey Muscle Tissue. Grilled turkey meat, in the form of small slices, was divided into portions and kept at −80 °C pending use in the experiment. This muscle tissue (1 g) was ground with 3 mL of liquid for 30 s in a laboratory blender (Waring, New Hartford, CT) and adjusted to pH 3.0. The liquid comprised SGF with red wine or ethanol solution or water as controls. The wine was diluted with 12% ethanol solution, so that the ethanol content (final concentration 6%) was the same in all of the treatments, except for the water control. The meat−liquid mixture in each treatment was divided among several tubes and incubated in a shaking bath at 37 °C for 180 min. At five time points, the hydroperoxides in the samples were extracted by serial 10-fold dilution in methanol under slow stirring for 15 min and filtered through no. 1442 filter paper (Whatman, England). One hundred microliter samples of the filtered solution were subjected to the FOX2 assay, which included TPP reagent controls, to prevent potential interference from ferric ions and the red wine pigments. Lipid peroxidation was also determined by the thiobarbituric acid according to the procedure described by Kanner and Harel (*35*).

Determination of Oxygen. The oxygen content in masticated food was monitored with an oxygen monitor (model 53, Yellow Spring Instrument Co., OH) with a Clark electrode. A piece of bread (5−20 g) was masticated and immediately placed into 100 mL of double-distilled water that had been deoxygenated by nitrogen bubbling, and the released oxygen was measured.

Cooxidation of Vitamins E and C and β-Carotene. The experimental system contained the ingredients found in the turkey muscle tissue, except that vitamin E, β-carotene, and vitamin C were introduced into the homogenate at concentrations of 25, 50, and 250 μM, respectively. Vitamin E was solubilized in ethanol and Tween 20 (0.05%). The preparation of β-carotene stock solution in water was as published previously (*32*).

Vitamin E Measurement. One volume of homogenate was mixed with nine volumes of ethanol, and after it was vortexed for 30 s, the mixture was centrifuged at 20800*g* for 3 min. The supernatant was filtered through a 0.2 μm membrane. A 20 μL sample was injected into a HPLC (Merck-Hitachi L-6200A) and separated with a Merck Lichrocart column PR-18, 125-4, eluted by an isocratic mobile phase of methanol at a flow rate of 1 mL/min and detected with a HPLC spectrofluorometer detector (Jasco FP 210). The element was excited at 290 nm and emitted at 329 nm. The results are the averages for three determinations for each sample. D-α-Tocopherol (Sigma) was used for calibration of a standard curve.

β-Carotene Measurement. The reaction in the homogenate was stopped by mixing two volumes of homogenate with one volume of hexane and one volume of ethanol. β-Carotene was extracted with the hexane upper phase and determined by spectral analysis at 460 nm. The extinction coefficient of β-carotene in hexane at 460 nm (for 1%, $A = 2550$) was used for calculation of carotenoid concentration.

Vitamin C Measurement. The homogenate samples were interacted with TCA (11.3%) (1:1 vol/vol) and centrifuged for 3 min at 20800*g*. The supernatant was filtered through a 0.2 μm membrane, and a 20 μL aliquot was injected into a HPLC (Merck-Hitachi L-6200A) and separated with a Merck Lichrocart column RP-18, 125-4, eluted with an isocratic mobile phase of KH_2PO_4 (10 mM):MeOH (97:3 vol/vol) and tetrabutylammonium hydroxide 0.75 mM, at a flow rate of 1 mL/min and detected with a diode array (Shimatzu, Kyoto) at 268 nm. AA (Merck) was used to generate a standard calibration curve.

Hydroperoxides and β-Carotene Measurement in Soybean Oil Emulsions (SBOs). Soybean oil was peroxidized by stirring and heating at 60 °C for 3 days. The reaction mixtures contained oil at 0.7 mg/mL, with 0.5% (v/v) Tween 20 and 3% (v/v) ethanol. The assay was similar to that for linoleic acid, except for the addition of a centrifugation step for 2 min at 20800*g* prior to the spectral analysis at 560 nm. β-Carotene was determined as described previously (*32*).

Determination of Red Wine Polyphenols. The polyphenols content of the wine was determined with Folin−Ciocalteau reagent and calculated as catechin equivalent (*33*). The results presented are the means of triplicates, and in the figures, each indicated error denotes the standard deviation.

Statistical Analysis. Results (means ± SD) are expressed as percentage or weight or molar basis. Statistical significance was assayed using one-way analysis of variance, following by a ranking procedure using Student−Newman−Keuls test (SAS software, SAS Institute Inc., Cary, NC). Results are the means of triplicates, and in the figures, each error bar (I) denotes the standard deviations.

RESULTS

Bread was masticated in the mouth of a volunteer for 10 s and introduced into 100 mL of deoxygenated water. The oxygen that diffused into the water from the bread was measured by an oxygen electrode. We found that 20 g of masticated bread was enough to increase the oxygen concentration in the water to ∼250 μM or full oxygen saturation (**Figure 1**).

Myoglobin and "free" iron ions are endogenous natural catalysts that are found in muscle food and that could accelerate lipid oxidation. HGF or SGF at pH 3.0 is a very good medium for lipid peroxidation of heated red meat; the reactions generate hydroperoxides and the genotoxic and cytotoxic compound, malondialdehyde (**Figure 2A**,**B**). Both reactions are inhibited by red wine polyphenols (**Figure 2A**,**B**). The cooxidation of vitamin E with red meat at pH 3.0 is presented in **Figure 3A**,**B**.

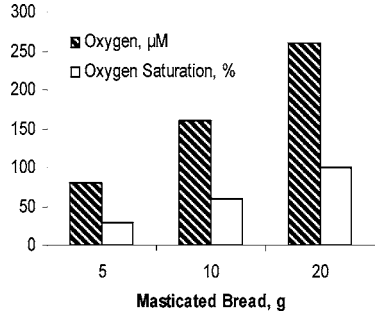

Figure 1. Oxygen evolution in deoxygenated water from masticated bread. The system contained bread masticated for 10 s and was introduced into 100 mL of deoxygenated water.

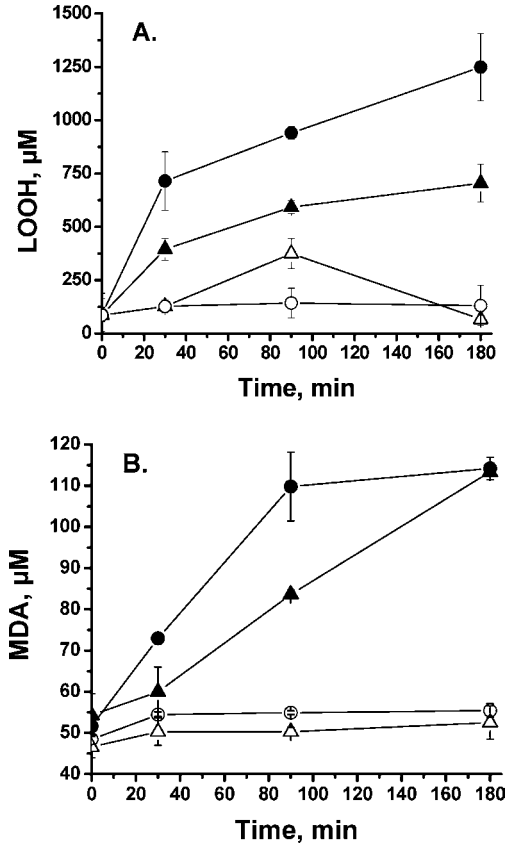

Figure 2. Lipid peroxidation of heated turkey muscle tissue in human and SGF at pH 3.0 incubated at 37 °C. The system contained heated ground turkey muscle tissue with three parts of human or SGF incubated at 37 °C. (**A**) Hydroperoxide accumulation; (**B**) thiobabituric acid reactive compounds as malondialdehyde. HGF (●); SGF (▲); HGF + red wine polyphenols (2.5 mM catechin equivalent) (○); and SGF + red wine polyphenols (2.5 mM catechin equivalent) (△).

During this reaction, the vitamin is oxidized completely in a few minutes. α-Tocopherol at 25 μM, which represents a plausible dietary concentration of ~10 mg/L, partially inhibited lipid peroxidation. Lipid peroxidation and the cooxidation of vitamin E were totally inhibited by red wine polyphenols at a concentration equivalent to 2.5 mM catechin.

The cooxidation of β-carotene in the presence of heated red meat at pH 3.0 is presented in **Figure 4**. β-Carotene at concentration of 18 and 50 μM inhibited lipid peroxidation only slightly. The coupled peroxidation reactions oxidized the provitamin to zero concentration. Both lipid peroxidation and β-carotene oxidation were inhibited by red wine polyphenols (**Figure 4A**,**B**).

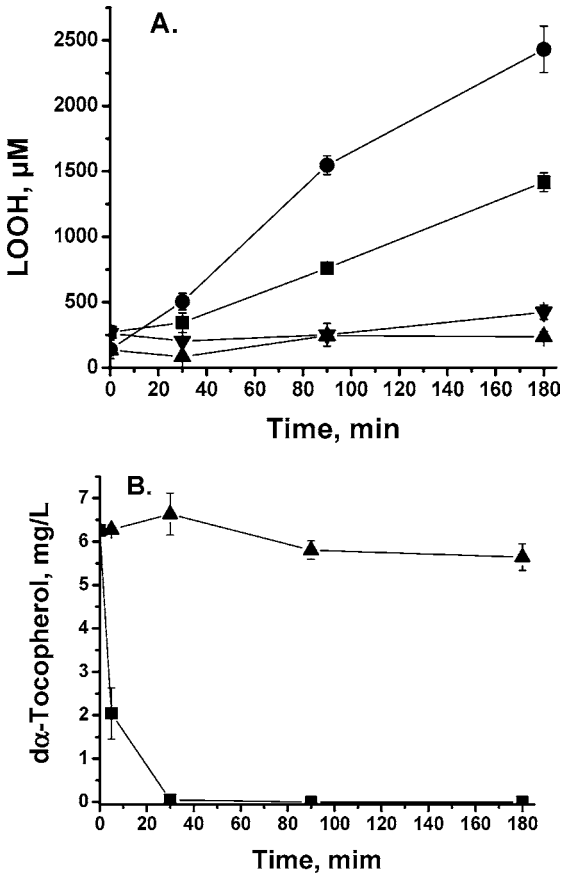

Figure 3. Cooxidation of α-tocopherol during lipid peroxidation of heated turkey muscle tissue in SGF at pH 3.0 incubated at 37 °C. The system contained heated ground turkey muscle tissue with three parts of HGF or SGF incubated at 37 °C; α-tocopherol was solubilized in ethanol solution and introduced into the muscle tissue homogenate. (**A**) Hydroperoxide accumulation; (**B**) α-tocopherol cooxidation. Control muscle tissue (●); muscle tissue + 14 μM α-tocopherol (■); muscle tissue + red wine polyphenols (2.5 mM catechin equivalent) (▼); and muscle tissue + 14 μM α-tocopherol + red wine polyphenols (2.5 mM catechin equivalent) (▲).

The possibility of a coupled peroxidation reaction was tested in the presence of AA also. AA at a concentration of 250 μM or 44 mg/L, which represents a possible concentration of the AA ingested during a regular meal, inhibited lipid peroxidation only slightly. The coupled peroxidation reaction oxidized AA (**Figure 5A**,**B**), but red wine polyphenols at a concentration of 2.5mM, which inhibited lipid peroxidation, did inhibit AA oxidation significantly but not totally.

In order to better understand the interaction between polyphenols and AA, we adopted a model system that we had used previously (*29*). Soybean oil in emulsion, containing 40 μM hydroperoxides and 15 μM β-carotene, was oxidized by metMb in SGF at pH 3.0. β-Carotene was used as a target molecule for the oxidation by the free radicals generated in this reaction. The introduction of catechin at a concentration of 10 μM inhibited this reaction only slightly. AA at 10 μM and 20 μM acted prooxidatively and increased the oxidation of β-carotene. However, in the presence of catechin (10 μM), AA at 10 μM and, especially, at 20 μM totally inhibited β-carotene oxidation, by working with catechin in a synergistic manner (**Figure 6**).

DISCUSSION

Recently, the Food and Nutrition Board of the USA National Academy of Sciences, Institute of Medicine, reported that there

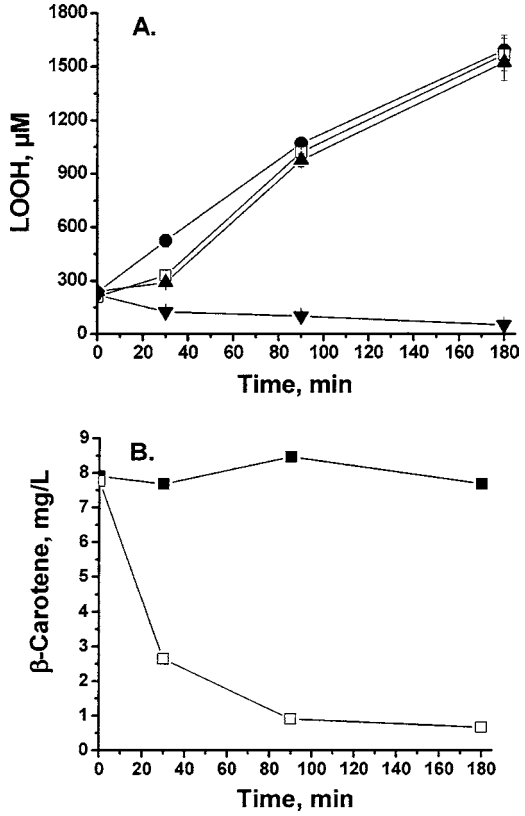

Figure 4. Cooxidation of β-carotene during lipid peroxidation of heated turkey muscle tissue in SGF at pH 3.0 incubated at 37 °C. The system contained heated ground turkey muscle tissue with three parts of HGF or SGF incubated at 37 °C; β-carotene was solubilized by 0.05% Tween 20 and introduced by this solution in the muscle homogenate. (**A**) Hydroperoxide accumulation; (**B**) β-carotene cooxidation. Control, muscle tissue (●); muscle tissue + β-carotene (18 μM) (□); muscle tissue + β-carotene (50 μM) (▲); muscle tissue + β-carotene (18 μM) + red wine polyphenols (2.5 mM as catechin equivalent) (■); muscle tissue + β-carotene (50 μM) + red wine polyphenols (2.5 mM as catechin equivalent) (▼).

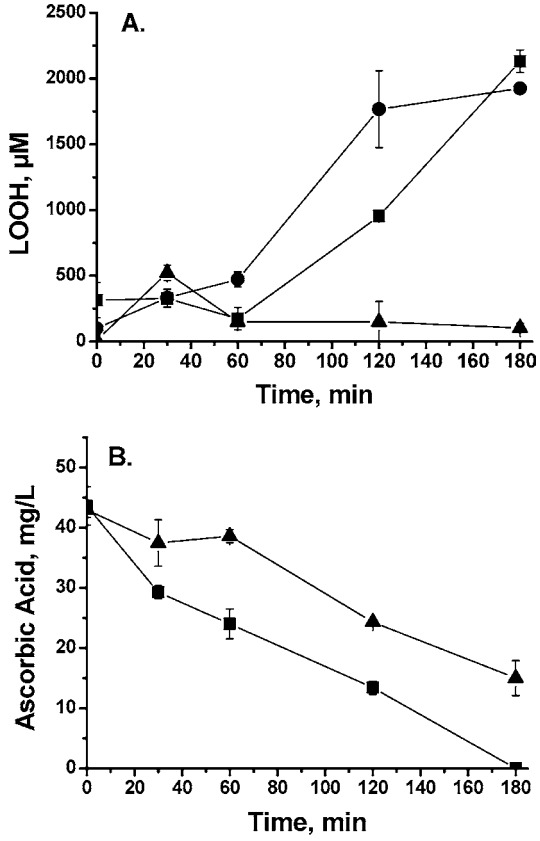

Figure 5. Cooxidation of AA during lipid peroxidation of heated turkey muscle tissue in SGF at pH 3.0 incubated at 37 °C. The system contained heated ground turkey muscle tissue with three parts of HGF or SGF incubated at 37 °C. (**A**) Hydroperoxide accumulation; (**B**) AA cooxidation. Control, muscle tissue (●); muscle tissue + AA (250 μM) (■); and muscle tissue + AA + red wine polyphenols (2.5 mM catechin equivalent) (▲).

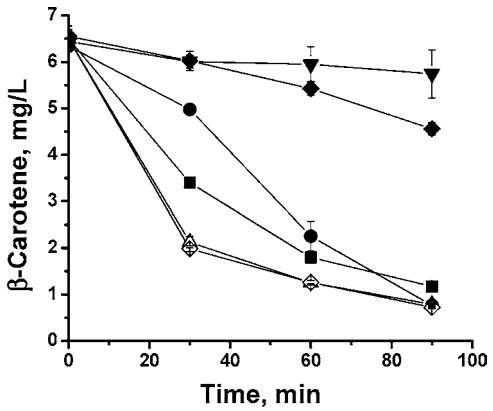

Figure 6. β-Carotene cooxidation during lipid peroxidation catalyzed by metMb in SBO, of SGF at pH 3.0 at 37 °C. Control SBO + metMb (20 μM) (■); SBO + metMb + AA (10 μM) (△); SBO + metMb + AA (20 μM) (◇); SBO + metMb + catechin (10 μM) (●); and SBO + metMb + catechin (10 μM) + AA (10 μM) (◆); SBO + metMb + catechin (10 μM) + AA (20 μM) (▼).

is insufficient evidence to support claims that eating megadoses of dietary antioxidants such as vitamins C and E or carotenoids, including β-carotene, can prevent chronic diseases (*34*). However, there is no doubt that all of these vitamins are very important for maintaining good health in humans, and the Board even increased the recommended daily allowance of vitamin C from 60 to 90 mg. Most of the vitamins, and especially the fat soluble vitamins, are recommended to be consumed with the meal. We have recently demonstrated that the stomach fluid is a good medium for further lipid peroxidation, which may affect vitamins or generate genotoxic and cytotoxic compounds (*29*). The stomach, which receives the masticated food and from time to time is open to the air, acts, at least during the meal time, in an aerobic environment. Our experiment with masticated bread demonstrated an easy way to achieve oxygen saturation in a deoxygenated solution.

Red meat, homogenized and incubated in HGF at pH 3.0, exhibited an autooxidation process that generated malondialdehyde and hydroperoxides. As muscle tissue contains free iron ions and myoglobin, both catalysts affect membranal lipid peroxidation in this system (*35*, *36*). Lipid peroxidation in both systems was inhibited by red wine polyphenols at the high concentration of 2.5 mM as catechin equivalent. This concentration is equivalent to 3-fold diluted red wine or two glasses of red wine (~300 mL) in a ~1000 mL stomach volume. Red meat, homogenized and incubated in HGF, underwent autooxidation similar to that which took place with SGF, which was subsequently used for the evaluation of the oxidation in the system.

Ten milligrams of D-α-tocopherol, which represents the recommended daily allowance of vitamin E, significantly inhibited red meat lipid peroxidation at pH 3.0. However, this reactivity decomposed the vitamin to zero content. α-Tocopherol, which is a lipophilic antioxidant, interacted very

efficiently with the peroxyl radicals formed in this system and so partly prevented further lipid peroxidation. Red wine polyphenols at an amount of 300 mL that simulated the consumption of wine during a meal inhibited both the lipid peroxidation and the coupled oxidation of vitamin E. β-Carotene is a lipophilic compound, which, at the same range of concentrations as α-tocopherol, inhibited lipid peroxidation very slightly, but its cooxidation was very rapid. Red wine polyphenols at a concentration of 2.5 mM (catechin equivalent) effectively inhibited the coupled oxidation of the vitamins. AA at a concentration that simulated the likely consumption of this vitamin during a meal only partially prevented lipid peroxidation. We hypothesize that the coupled oxidation of AA in this system was lower than that of vitamin E or β-carotene, most probably because of its hydrophilic properties. As its reducing power is higher than that of most of the red wine polyphenols and because polyphenols are more lipophilic than AA, red wine phenoxyl radicals generated during interaction with lipid radicals were reduced by AA and not vice versa (*37*−*40*). For this reason, the potential of red wine polyphenols to prevent AA oxidation was only partially realized.

The special interrelations between a polyphenol, catechin, and AA with relation to lipid peroxidation were demonstrated at the low pH in the present study. Catechin at 10 μM, which showed only a very slight antioxidant effect, was synergistically affected by AA (which, when alone, acted prooxidatively). We hypothesize that the synergistic effect was achieved through a reaction by which AA regenerated oxidized catechin to a reduced form, which was a more effective antioxidant for lipids, via reaction 1.

$$AH_2 + PhO^{\bullet} \rightarrow AH^{\bullet} + PhOH \quad (1)$$

In the present study, we demonstrated that the oxidation of foods such as red turkey meat, in simulated or actual HGF, in addition to generating hydroperoxides and other cytotoxic compounds, also oxidized antioxidant vitamins. Plant antioxidants such as red wine polyphenols prevented the buildup of oxidized lipid products and destruction of vitamin E and β-carotene but vitamin C to a lesser extent. The AA found in foods could enhance the activity of the polyphenols through a synergistic antioxidant effect. A high concentration of vitamin C in the stomach during the meal could increase the net work activity of the polyphenols and so better protect other essential dietary constituents from cooxidation. Prevention of lipid peroxidation in the stomach by food-derived polyphenols seems to increase the overall amount of antioxidant vitamins that arrive in the gastrointestinal tract for further absorption into the blood stream.

The present data may explain the major benefits and the crucial role of the consumption of food products rich in dietary antioxidants during the meal. It seems that cooked red meat and heated frying oils are prone to enhanced lipid peroxidation in the gastric medium. This reaction could be very efficiently inhibited by the antioxidant network provided by the reducing compounds found in foods. These compounds include AA, glutathione, and polyphenols, which occur in complex mixtures; they most probably work in the stomach during the meal as part of the antioxidant network provided by vegetables, fruits, and beverages such as red wine, tea, cocoa, or coffee. It seems to us that the antioxidant network in the stomach could indirectly decrease hydroperoxides and other cytotoxic compounds and, in parallel, increase the vitamin antioxidants that reach the blood system. This trend would have a synergistic effect on the antioxidative tone of the plasma. The balance between prooxidation and antioxidation in the stomach seems to have a very important impact on our health (*29*). This may help better to explain the "French paradox" and the effect of Mediterranean and Japanese diets, in which products with high antioxidant content such as polyphenols are consumed during the meal. However, we need more information about the reactions of these constituents in the human stomach and other parts of the gastrointestinal tract (*41*) in vivo, in order further evaluate the potential roles of dietary fats, food endogenous catalysts, and plant-derived antioxidants in atherogenesis and other diseases.

NOTE ADDED AFTER ASAP PUBLICATION

Figure 4 and its caption were incorrect in the original ASAP posting of April 6, 2005. The corrected figure and caption are shown in the posting of April 8, 2005.

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