The 6-a-day study: effects of fruit and vegetables on markers of oxidative stress and antioxidative defense in healthy nonsmokers[1–4]

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ABSTRACT

Background: Fruit and vegetables contain both nutritive and nonnutritive factors that might contribute to redox (antioxidant and prooxidant) actions.

Objective: We investigated the relative influence of nutritive and nonnutritive factors in fruit and vegetables on oxidative damage and enzymatic defense.

Design: A 25-d intervention study with complete control of dietary intake was performed in 43 healthy male and female nonsmokers who were randomly assigned to 1 of 3 groups. In addition to a basic diet devoid of fruit and vegetables, the fruit and vegetables (Fruveg) group received 600 g fruit and vegetables/d; the placebo group received a placebo pill, and the supplement group received a vitamin pill designed to contain vitamins and minerals corresponding to those in 600 g fruit and vegetables. Biomarkers of oxidative damage to protein and lipids and of antioxidant nutrients and defense enzymes were determined before and during intervention.

Results: Plasma lipid oxidation lag times increased during intervention in the Fruveg and supplement groups, and the increase was significantly higher in the former. Plasma protein carbonyl formation at lysine residues also increased in both of these groups. Glutathione peroxidase activity increased in the Fruveg group only. Other markers of oxidative damage, oxidative capacity, or antioxidant defense were largely unaffected by the intervention.

Conclusions: Fruit and vegetables increase erythrocyte glutathione peroxidase activity and resistance of plasma lipoproteins to oxidation more efficiently than do the vitamins and minerals that fruit and vegetables are known to contain. Plasma protein carbonyl formation at lysine residues increases because of the vitamins and minerals in fruit and vegetables. *Am J Clin Nutr* 2004;79:1060–72.

KEY WORDS Fruit, vegetables, human intervention, lipoprotein oxidation, protein carbonyls, glutathione peroxidase, glutathione *S*-transferase, antioxidant capacity

INTRODUCTION

Protective actions of fruit and vegetables against chronic diseases have been reported within several populations, including Finnish (1, 2), Italian (3), Dutch (4), Japanese (5), British (6, 7), and North American (8, 9) populations. The diversity of dietary preferences for various fruits and vegetables within these populations has led to public campaigns for a general increase in the consumption of these food items, such as the 5-a-day campaign in several countries, including the United States (10, 11). In Denmark, a 6-a-day campaign recommending that the public increase its intake of fruit and vegetables was launched 4 y ago; the aim of the campaign was a 600-g/d intake in adults (12).

The mechanisms behind the protective actions of fruit and vegetables are not well known. One hypothesis is that fruit and vegetables contain factors that strengthen our defense against reactive molecules (13). A prominent hypothesis is based on the potential antioxidant effect of many nutrients and nonnutrients in plant foods (14, 15). According to this hypothesis, the chronic intake of dietary antioxidants from fruit and vegetables would lead to a sustained decrease in oxidative damage to key structures in the body, including lipids, proteins, and DNA. However, intervention with purified antioxidative nutrients, such as vitamin C, α-tocopherol, and β-carotene, did not prove to be generally protective against chronic diseases (16–18). Relatively weak or conflicting short-term effects of these nutrients and of vitamin C on markers of oxidative damage have been observed in humans (19, 20). However, a few fruit and vegetable intervention studies do point toward effects on lipid oxidation (21, 22).

Another prominent hypothesis concerns induction of defense enzymes. This has been supported by findings of genetic

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response elements that interact with xenobiotics and redox-active compounds and lead to increased concentrations of enzymes such as quinone reductase and glutathione *S*-transferases (GSTs) (23, 24). Many chemically diverse compounds in plants have subsequently been observed to induce defense enzymes in cell culture studies (25–27). Human intervention studies with high amounts of plant foods rich in these factors have shown that protective enzyme induction may also take place in humans (28). The regulation of defense enzymes involved in removal of reactive oxygen species is less well known. Although chemical and physical inducers of such enzymes are known (29–31), the presence of dietary effects on the activation or induction of antioxidant enzymes is controversial (32–35).

To our knowledge, the question of whether nutrients or nonnutrients act as mediators of protection has not been addressed previously in an intervention study. The present trial was therefore designed within the 6-a-day concept to address this question and whether enzyme induction or antioxidative effects are mechanistically involved. Moreover, because we previously observed an apparent prooxidant effect of fruit and vegetables toward proteins in several crossover intervention studies (32, 33, 36), we included a postperiod to observe the time course for several biomarkers to return to their initial values.

SUBJECTS AND METHODS

Subjects

Healthy, normal-weight men and women were recruited for the study by advertisement at local universities and other local institutions and in the local newspaper. Exclusion criteria were smoking, obesity, family history of chronic diseases, hypertension, use of any medication, and heavy physical exercise. The aim of the study was fully explained to the subjects before they gave their written consent. Forty-eight subjects were recruited, and 43 completed the study. One subject dropped out because he was unable to be present for lunch within a specific time interval, and 4 subjects dropped out because of events unrelated to the study. The research protocol was approved by the Scientific Ethics Committee of the municipalities of Copenhagen and Frederiksberg (01-234/99).

Study design

The study was a randomized, partly blinded intervention experiment with complete dietary control and a parallel design. Subjects were asked at recruitment to complete a previously validated food-frequency questionnaire (37), and a 4-d weighed food record was performed during run-in. At recruitment, each subject was randomly assigned to 1 of 3 groups, all of which were served a basic diet free from fruit and vegetables for 24 d. The diets were supplemented with either 600 g fruit and vegetables (Fruveg group, $n = 16$), a vitamin and mineral supplement (supplement group, $n = 12$), or a placebo supplement (placebo group, $n = 15$); for details see the Diets subsection below. The diets were coded with different colors, and the codes were not broken until the biomarker analyses were completed and the results were analyzed statistically. The study was double-blinded with respect to the placebo and supplement groups, but it was not possible to blind the subjects to the intake of fruit and vegetables. However, the analyzers were blinded to all blood and urine samples. The subjects were carefully instructed not to change their physical activity level during the study. For logistic reasons, the study had to be split into 4 sections, which were conducted during the months of January–May, with 9, 7, 13, and 14 volunteers in each of the 4 sections.

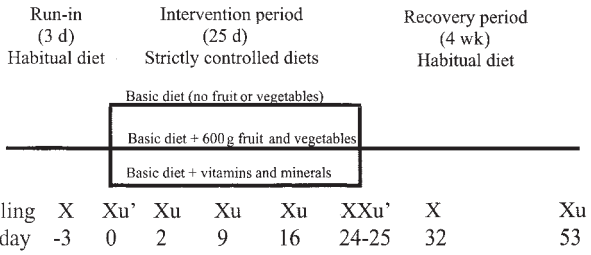

FIGURE 1. Graphic presentation of the overall design. During the intervention period, the subjects were divided into 3 groups who followed a strictly controlled dietary regimen. The first group ($n = 15$) was given a basic diet with no fruit and vegetables. This group was also given an energy drink and a placebo tablet. The second group ($n = 16$) was given the same basic diet but with an additional 600 g fruit and vegetables; all fruits and vegetables were given in proportion to median Danish intakes. The third group ($n = 12$) was given the basic diet but also received the energy drink and a vitamin and mineral tablet to compensate for the nutritional content of the fruit and vegetables consumed by the second group. X, fasting blood samples; ', postprandial blood samples; u, 24-h urine sample.

The subjects received all foods and drinks from the Research Department of Human Nutrition at the Royal Veterinary and Agricultural University, Frederiksberg, Denmark, and were not allowed to consume other foods in the study periods, except for water and salt. On weekdays, lunch was consumed in the department under supervision, whereas beverages, dinner, snack, and breakfast for the next morning were provided daily as a package with guidelines for preparation and consumption. Food and beverages for the weekend were provided on Fridays. Leftovers were brought back to the department for registration.

Fasting blood samples were taken in the morning before breakfast to reflect the previous day and are termed according to the day that they reflect. Collections were performed twice before the intervention period (run-in, days −3 and 0); on days 2, 9, and 16 of the intervention period; twice at the end of the intervention period (days 24 and 25); and 1 wk (day 33) and 4 wk (follow-up, day 53) after the end of the intervention when the participants had resumed their habitual diets. The end samples, ie, those from days 24 and 25, were thus taken at the midpoint in time between the run-in and the follow-up samples to allow for a better possibility of observing time trends unrelated to the intervention. Postprandial blood samples were also collected 16 h before the fasting samples were taken at commencement (day −3) and on the last trial day (day 25) to test whether certain short-term effects of the diets could be observed.

Twenty-four–hour urine samples were collected at commencement (day 0), at 4 occasions during the intervention period, and at follow-up 4 wk after the end of intervention. The overall design and sampling scheme are shown in **Figure 1**.

Diets

The basic part of the diets served to all 3 groups consisted of 5 menus repeated in a 7-d cycle (**Table 1**), and these menus consisted of ordinary Danish meals. Most food was prepared in one batch in the kitchen, weighed, and frozen at −20 °C until use. The Fruveg group received an additional 600 g fruit and vegetables that reflected current preferences of Danish consumers

TABLE 1
Composition of experimental diet at an energy intake of 10 MJ/d

Meal and component	Menu 1	Menu 2	Menu 3[1]	Menu 4[2]	Menu 5[3]
			g fresh wt/d		
Breakfast					
Whole-grain bread	70	70	70	70	70
Cheese	25	25	25	25	25
Sour milk	—	195	195	—	—
Rye bread crumbs	—	33	33	—	—
Sugar	14	—	—	14	14
Oats	45	—	—	45	45
Defatted milk	135	—	—	135	135
Lunch					
Rye bread	100	100	100	100	100
Roast beef	—	28	—	28	—
Tuna salad	—	—	—	50	—
Egg salad	46	—	—	—	46
Ham	28	—	—	—	28
Ham salad	—	50	—	—	—
Turkey	—	—	28	—	—
Mackerel salad	—	—	50	—	—
Dinner					
Meat sauce	—	—	—	190	—
Beef stew	200	—	—	—	220
Pasta	—	70	—	70	—
Rye bread	—	—	40	—	—
Rice	70	—	—	—	70
Cheese sauce	—	200	—	—	—
Pork stew	—	—	450	—	—
Snack					
Whole-grain bread	—	70	—	70	—
Cheese spread	20	—	—	20	—
Sponge cake	110	—	110	—	110
Mazarin cake	—	50.3	—	50.3	—
Defatted milk	250	250	250	250	250

[1] Repeated on day 5 of each week during the intervention.
[2] Repeated on day 7 of each week during the intervention.
[3] Almost identical to menu 1 and served on day 6 of each week during the intervention.

(**Table 2**). The relative contents of stone fruits, citrus fruits, roots, cruciferous vegetables, *Allium* vegetables, and others were assessed from a recent Danish dietary survey (NL Andersen, Danish Institute for Food and Veterinary Research, personal communication, 2001) and were scaled up proportionally to a total weight of 600 g. To simplify the diets, stone fruits were represented by apples and pears, which are the preferred items within this fruit group in Denmark. Similarly, orange juice represented citrus fruits; carrot, roots; broccoli, cruciferous vegetables; onion, *Allium* vegetables; and tomato, others. For the Fruveg group, canned tomatoes (100 g) and fresh onions (25 g) were incorporated into the hot dinner meal, fresh carrots (75 g) and broccoli (100 g) were served as additional items at lunch, and apples (90 g), pears (90 g), and orange juice (120 g) were combined into a fruit salad, of which half was served in the morning and the other half in the evening. This pattern was repeated on each day of the intervention in the Fruveg group and was independent of the basic menu (Table 1). A vitamin and mineral tablet (manufactured and provided by Pharma Vinci, Frederiksværk, Denmark) was designed to contain one-third of the known antioxidant vitamins and minerals that were calculated to be present in the 600 g fruit and vegetables by using a computer-based program (DANKOST; Danish Catering Center, Herlev, Denmark) based on the food-composition database of the Danish Veterinary and Food Administration (38). The tablet was given to the subjects in the supplement group with each meal so that they received 3 tablets/d (**Table 3**). To ensure that the subjects in the supplement and Fruveg groups received the same amount of bioavailable folate and β-carotene, the folate and β-carotene contents in the 3 pills were only 50% of the calculated contents in the fruit and vegetables. A placebo tablet (Pharma Vinci) with identical appearance was given to the subjects in the placebo group. The supplement and placebo groups consumed an additional "energy drink" containing simple sugars (11.3 g glucose, 20 g fructose, 27.9 g sucrose, 1.5 g citric acid, 350 mL water, and 1189 kJ total energy) to balance the extra energy intake due to the 600 g fruit and vegetables in the Fruveg group. Thus, all the subjects received approximately the same amounts of carbohydrate, fat, protein, and total energy per kilogram of body weight.

TABLE 2
Daily additions of items to the diet of the fruit and vegetables group

Meal and component	Menus 1–5
	g fresh wt/d
Breakfast	
Orange juice	60
Apple	45
Pear	45
Sugar	5
Lunch	
Broccoli	100
Carrot	75
Dinner	
Onion	25
Tomato	100
Orange juice	60
Apple	45
Pear	45
Sugar	5

TABLE 3
Daily additions of vitamins and minerals to the diet of the supplement group

Vitamin or mineral	Declared[1]	Analyzed
Calcium, total (mg/d)	180	—
Copper, total (mg/d)	0.4	—
Iron, total (mg/d)	3	—
Magnesium, total (mg/d)	65	—
Manganese, total (mg/d)	1.1	—
Phosphorous, total (mg/d)	150	—
Potassium, as KCl (mg/d)	1200	—
Zinc, total (mg/d)	1.4	—
β-Carotene (mg/d)	5.0	—
Thiamine (mg/d)	0.3	0.31
Riboflavin (mg/d)	0.4	0.40
Vitamin B-6 (mg/d)	0.6	0.53
Niacin (mg/d)	3	—
Pantothenic acid (mg/d)	1.7	—
Biotin (μg/d)	1.7	—
Folate (μg/d)	125	—
Vitamin C (mg/d)	150	141
α-Tocopherol (mg/d)	3.3	3.5
Vitamin K_1 (μg/d)	250	—

[1] By the manufacturer (Pharma Vinci, Fredericksværk, Denmark).

TABLE 4
Characteristics of the diets consumed by the 3 groups[1]

	Fruveg group	Supplement group	Placebo group
Carotenoids, total (mg/d)	18.1	5.8	0.8
β-Carotene (mg/d)	10.8	5.2	0.2
Carbohydrate, total (% of energy)	55	57	57
Fat (% of energy)	27	27	26
Saturated (g/d)	28.8	27.1	27.1
Monounsaturated (g/d)	30.2	29.9	29.9
Polyunsaturated (g/d)	15.5	15.0	15.0
Fiber (mg/d)			
Total	38.2	30.5	30.5
Soluble	13.4	10.5	10.5
Folate (μg/d)	450	320	195
Iron, total (mg/d)	15	15	12
Magnesium, total (mg/d)	389	387	322
Manganese, total (mg/d)	7.8	7.7	6.6
Protein, total (% of energy)	16	15	15
Thiamine (mg/d)	1.8	1.8	1.5
Riboflavin (mg/d)	2.2	2.2	1.8
Vitamin B-6 (mg/d)	2.0	2.0	1.4
Vitamin C (mg/d)	220	173	23
Vitamin E (mg/d)	8	8	5
Zinc, total (mg/d)	15.0	15.0	13.6

[1] All values are $\bar{x}$ per 10 MJ. Fruveg, fruit and vegetables. Values for the following were analyzed: total carotenoids; β-carotene; total carbohydrate; fat; saturated, monounsaturated, and polyunsaturated fat; total and soluble fiber; folate; total protein; vitamin C; and vitamin E. Values for the following were calculated by using the DANKOST2000 calculation program (Danish Catering Center, Herlev, Denmark) based on the food-composition database of the Danish Veterinary and Food Administration (38): total iron, total magnesium, total magnanese, thiamine, riboflavin, vitamin B-6, and total zinc.

The manufacturer kept the code for the tablet identities until completion of biochemical and statistical analyses.

The subjects were not allowed to drink coffee, tea, chocolate, or alcoholic beverages. The energy requirements of the subjects were estimated on the basis of body weight, age, and physical activity and were slightly higher than the energy intakes calculated from the food-frequency questionnaire. Individual portions were prepared according to estimated energy requirements at the Research Department of Human Nutrition. Body weight was measured every second day, and an average weight variation of >1 kg resulted in either the allocation to a different energy intake or the requirement to consume extra rolls (same macronutrient composition as the total diet). The mean energy intake during the study was 13 MJ (range: 9–17 MJ). Consumption of extra rolls was noted in a diary, as were any signs of illness, medications, and any deviation from the diet.

Food analyses

The calculated and analyzed contents of macronutrients and selected vitamins and minerals in the 3 diets are presented in **Table 4**. Duplicate portions of the diets with or without fruit and vegetables were taken on 2 occasions to analyze for the dry weight; the content of carotenoids, dietary fiber, total nitrogen, and fats; and the fatty acid composition. On 4 occasions, duplicate portions of the relevant food items were collected for analysis of folate and flavonoids. These samples were pooled to represent the average weekly diets and were then analyzed. Nitrogen was determined according to the principle of Dumas (39) on an automatic nitrogen analyzer (NA 1500; Carlo Erba Strumentazione, Milano, Italy). The content of dietary fiber was determined with the use of gas chromatography after enzymatic digestion (40). The fatty acid composition of the diets was analyzed by gas chromatography (model 8420; PerkinElmer, Shelton, CT) after extraction and methylation with methanolic boron trifluoride (41). The carotenoids lutein, zeaxanthin, lycopene, α-carotene, and β-carotene were determined in 10-g homogenized whole-meal samples as described previously (42). Vitamin C was determined in 30–100-g whole-meal samples (43). Flavonoids were determined in 10-g samples of broccoli and fruit salad as described previously (44) and in 20-g portions of all the other samples of relevant food items.

A modified version of the microbiological method in *Pharmacopoea Nordica* (45) was used for the determination of folate. A whole-meal sample (5 g) was homogenized with 100 mL 1% ascorbic acid:1 mmol phosphate buffer (pH 6.0)/L and was subsequently autoclaved for 5 min at 121 °C. This solution was diluted to 20 mL. For deconjugation, 7 mL was incubated for 60 min at 37 °C after addition of 100 μL of a solution of one Pancrex-V capsule (Paines & Byrne Limited, Greenford, United Kingdom) in 5 mL water and of 100 μL of 1 mL freeze-dried human plasma powder (P9523; Sigma, St Louis) dissolved in 1 mL 1 mol L-cystein hydrochloride/L (Merck, Darmstadt, Germany) in water. The enzyme activity was stopped by autoclaving for 5 min at 121 °C. The solution was diluted to 100 mL with 1% ascorbic acid:1 mmol phosphate buffer (pH 6.0)/L. Aliquots of this solution were incubated with *Lactobacillus casei* for 24 h at 37 °C and compared with processed standards of folic acid (no. 388019; Centre for Chemical Reference Substances, Stockholm) by turbidometric measurement at 650 nm (UV1601; Shimadzu, Kyoto, Japan).

Blood and urine sampling and preanalytic sample handling

After the subjects rested in a supine position for 10 min, their fasting blood samples were collected in EDTA-coated tubes for analyses of vitamin C, ferric-reducing ability of plasma (FRAP), Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carbonic acid; Aldrich Chemical Co, Steinheim, Germany)-equivalent antioxidant capacity (TEAC), lipoprotein oxidation lag time, plasma malondialdehyde (MDA), MDA in LDL (LDL-MDA), plasma protein carbonyls at lysine residues (amino adipic semialdehyde, AAS), total carbonyls in specific plasma proteins, antioxidant enzymes, and GST. Blood samples were collected in sterile tubes and allowed to stand at room temperature to collect serum for analysis of carotenoids, retinol, and α-tocopherol.

Isolation of LDL from plasma (200 μL) was done by density gradient ultracentrifugation at 220 000 × *g* for 18 h at 4 °C (46). To prevent oxidation, 0.25 mmol EDTA/L and 0.1 mmol butylated hydroxytoluene/L were added to the density solution.

For analysis of vitamin C, blood samples were immediately centrifuged at 1500 × *g* for 10 min at 4° C, and the plasma was mixed 1:1 with 10% meta-phosphoric acid. The samples were stored at −80 °C for a maximum of 3 mo before analysis of vitamin C. The remainder of the fresh blood was allowed to stand for coagulation, and serum was collected for the analysis of vitamins A and E and carotenoids.

Twenty-four–hour urine samples were collected on 50 mL of 1 mol HCl/L in 2.5 L polyethylene flasks with tight screw-caps. An extra 0.5-L flask containing 10 mL 1 mol HCl/L and 2 mL 10% ascorbate was also given to the participants to help them in the collection of larger volumes. Females were given a funnel with a wide opening (25 cm) to help them collect their urine without spills. The samples from the 2 flasks were mixed, and aliquots were stored at −80 °C. Aliquots were shipped on dry ice and kept frozen at −70 °C until analysis.

Micronutrients in the blood compartment

Analyses of vitamin C in plasma (43) and of carotenes and tocopherol (47) in serum were performed as previously described. The interday CVs for the 3 analyses were 9.7%, 9.2%, and 10.1%, respectively.

Biomarkers of plasma lipid and protein oxidation

Plasma lipoprotein oxidation lag time and plasma lysine carbonyls (AAS) were determined as described previously (48). The interday CVs were 5.6% and 12.2%, respectively (49). Total carbonyls in selected plasma proteins in plasma samples from the run-in period and the end of the intervention were determined by using a semiquantitative electrophoretic technique (50).

Total plasma MDA and LDL-MDA were determined by using a modified version of the HPLC method of Lauridsen and Mortensen (51) and Cighetti et al (52). The antioxidant butylated hydroxytoluene was added to 70 μL plasma or LDL samples to give a final concentration of 2 mmol/L, and the samples were hydrolyzed by adding acetic acid (final concentration of 2.6 mol/L) and heating for 30 min at 60 °C. To these samples, 100 μL trichloroacetic acid was added, and the samples were mixed and centrifuged for 5 min at 13 000 × *g* and room temperature. To 100 μL of the supernatant fluid, 100 μL 2-thiobarbituric acid was added, and the sample was heated at 95 °C for 1 h. For the HPLC analysis, 40 μL was injected with a gradient of aqueous 0.1% trifluoroacetic acid:acetonitrile (0–3.9 min, 0–40%; 3.9–4 min, 40–100%; 6 min, 100%). The HPLC analysis was performed on a Hewlett-Packard 1090 system [Agilent (formerly Hewlett-Packard), Waldbronn, Germany] with a diode array detector (detection at 532 nm) and a Zorbax SB-C_{18} column (4 × 150 mm, 3.5 μm; Agilent). Five MDA standards in plasma and an assay blank were included in each run. The concentration of MDA was calculated from the linear standard curve in spiked plasma (range: 0–135 pmol MDA/mg protein). The limit of detection was <1 pmol/mg protein, and the interassay CVs were 25% for plasma MDA and 36% for LDL-MDA.

MDA was purchased from Aldrich Chemical Co, butylated hydroxytoluene and 2-thiobarbituric acid were purchased from Sigma Chemical Co, and trichloroacetic acid was purchased from Riedel-de Haën (Seelze, Germany). Trifluoracetic acid was purchased from Merck-Schuchardt (Hohenbrunn, Germany), acetic acid was purchased from Merck, and acetonitrile, which was HPLC grade, was purchased from Rathburne (Walkerburn, United Kingdom).

Isoprostanes in urine

The urinary samples from this study were analyzed for 8-isoprostane $F_{2\alpha}$ (8-*iso*-$PF_{2\alpha}$) by radioimmunoassay as previously described (53). In brief, an antibody was raised in rabbits by immunization with 8-*iso*-$PF_{2\alpha}$ coupled to bovine serum albumin at the carboxylic acid by the 1,1'-carbonyldiimmidazole method. The cross-reactivity of the antibody with 8-*iso*-15-keto-13,14-dihydro-$PF_{2}\alpha$, 8-*iso*-$PF_{2\beta}$, $PF_{2\alpha}$, 15-keto-$PF_{2\alpha}$, 15-keto-13,14-dihydro-$PF_{2\alpha}$, thromboxane B2, 11β-$PF_{2\alpha}$, 9β-$PF_{2\alpha}$, and 8-*iso*-$PF_{3\alpha}$ was 1.7%, 9.8%, 1.1%, 0.01%, 0.01%, 0.1%, 0.03%, 1.8%, and 0.6%, respectively. The detection limit of the assay was ≈23 pmol/L, and the interday CV was 8.5%. The acidified urinary samples were thawed, and 50 μL was used in the assay. The total excretion of urinary 8-*iso*-$PF_{2\alpha}$ was calculated on the basis of the 24-h diuresis.

Antioxidant capacity markers

The FRAP assay (54) and the TEAC assay (55) were automated on a Rosys Plato 3000 (Immucor Gamma, Norcross, GA) with the use of flat-bottom, 96-well microtiter plates (Nunc Maxisorp; Nalge Nunc International, Rochester, NY). Interday CVs for the FRAP and TEAC assays were 8.8% and 16.6%, respectively. All chemicals were from Merck unless stated otherwise. For the analysis of FRAP, 10 μL plasma was added along with 25 μL water and 265 μL freshly prepared FRAP reagent (2,4,6-tripyridyl-s-triazine; Sigma). The plate was incubated for 4 min at 37 °C, and the absorbance was read at 620 nm (A_1). Blanks (water replacing plasma) were placed diagonally across the plate, and a mean absorbance was calculated (A_2). For each sample, the difference in absorbance at 620 nm between samples and blanks ($\Delta A_{620} = A_1 - A_2$) was calculated and related to the ΔA_{620} of processed Fe(II) standard solutions (8 concentrations in the range of 20–5000 μmol $FeSO_4$/L; $n = 3$) from the same plate.

For analysis of TEAC, manganese dioxide (Aldrich Chemical Co) was added in excess to an aqueous solution containing 35 mmol diammonium 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonate)/L (ABTS; Fluka Chemie, Buchs, Switzerland) to create an ABTS-radical solution. The mixture was passed through a 0.2-μm Minisart filter (Sartorius, Göttingen, Germany) to remove excess manganese dioxide, and the solution was diluted 100 times in water to an absorbance of 1.5 at 405 nm (Shimadzu UV-160 spectrophotometer). The ABTS-radical solution was evaporated to dryness under a vacuum in portions of 1 mL and stored at −20 °C until use. Before use an ABTS-radical working solution was prepared by dissolving one ampoule of ABTS-radicals in 10 mL 50 mmol phosphate-buffered saline (PBS)/L. To 5 μL plasma, 270 μL 50 mmol PBS/L was added, the absorbance was read at 620 nm (A_1), and the plate was incubated for 10 min at 37 °C. Then, 30 μL ABTS-radical working solution was added, and the plate was incubated for an additional 2.5 min at 37 °C before the second reading (A_2). Blanks (PBS) were placed diagonally across the plate, and a mean was calculated (B). A 2.5-mmol Trolox/L solution was prepared in PBS and used as a stock standard. The absorbance change was calculated for each sample [$\Delta A_{620} = B - (A_2 - A_1)$] and related to the dose-response curve of Trolox (0–2.5 mmol Trolox/L) present on each plate.

Oxygen consumption was determined as described previously (36), and the observed intraday CV in the present study was 31.4%. TEAC, FRAP, and oxygen consumption were determined on postprandial plasma samples to test whether short-term effects of the diets could be observed. TEAC and FRAP were additionally determined in fasting plasma samples.

TABLE 5
Characteristics and daily dietary habitual intakes in the 3 groups at the start of the study[1]

	Fruveg group (n = 8 M, 8 F)	Supplement group (n = 7 M, 5 F)	Placebo group (n = 7 M, 8 F)
Age (y)	29 ± 8	24 ± 4	26 ± 6
BMI (kg/m^2)	23.5 ± 2.3	23.1 ± 1.7	22.6 ± 2.3
Energy intake (kJ)	9920 ± 2220	9810 ± 4070	9260 ± 3330
Sugar (g)	67 ± 42	57 ± 37	76 ± 76
Dietary fiber (g)	20 ± 7.3	16 ± 7	14 ± 7
Alcohol (% of energy)	13 ± 16	16 ± 19	17 ± 18
Protein (% of energy)	17 ± 3	17 ± 3	15 ± 3
Fat (% of energy)	29 ± 6	31 ± 3	29 ± 6
Carbohydrate (% of energy)	41 ± 8	36 ± 6	39 ± 8
Fruit and vegetables (g)	330 ± 180	270 ± 120	260 ± 180
Folate (μg)	330 ± 80	300 ± 130	260 ± 90
Iron (mg)	12 ± 3	12 ± 5	11 ± 4
Vitamin A (mg)	1330 ± 390	1350 ± 9502	990 ± 330
Vitamin C (mg)	83 ± 32	65 ± 33	72 ± 46
Vitamin D (μg)	5 ± 2	4 ± 2	4 ± 1
Vitamin E (mg)	7 ± 2	6 ± 2	6 ± 2
β-Carotene (mg)	2700 ± 1200	2300 ± 900	2000 ± 1000

[1] All values are $\bar{x}$ ± SD. Values were calculated by using the DANKOST2000 calculation program (Danish Catering Center, Herlev, Denmark) based on the food-composition database of the Danish Veterinary and Food Administration (38). Dropouts were omitted from the analysis but did not differ from the other subjects. Fruveg, fruit and vegetables. There were no significant differences between the groups.

Defense enzyme activities

Erythrocyte glutathione reductase, glutathione peroxidase (Gpx), catalase, and superoxide dismutase were determined as previously described (36). Their intraday CVs were 6.8%, 9.7%, 16.4%, and 12.8%, respectively.

Erythrocyte GST was determined on a Cobas Mira analyzer according to Habig et al (56) with modifications. Briefly, the erythrocyte lysates were diluted 1:3 in 150 mmol KCl/L containing 2 mmol EDTA/L and 0.1% Triton X-100 (pH 7.0). A total of 6 μL lysate was transferred to a reaction mixture containing 484 μL 1 mmol reduced glutathione/L in 100 mmol phosphate buffer (KH_2PO_4)/L and 10 μL 1-chloro-2,4-dinitrobenzene (Riedel-de Haën) in dimethylsulfoxide. All samples were run in duplicate. The enzymatic activities were calculated relative to the amount of hemoglobin, which was measured with the use of a standard kit and Drabkins reagent (Randox Laboratories Ltd, Crumlin, United Kingdom). The intraday CV was 7.4%.

Statistics

Data were analyzed for homogeneity of variance by using Levenes test and for normal distribution by using Shapiro-Wilks W-test, both with a P value of 0.05. Data that could not meet these criteria were transformed logarithmically. Data from days that did not meet the criteria after transformation were omitted from the subsequent analysis of variance and t test analyses and were analyzed by nonparametric tests. To assess efficiency of randomization, differences between the run-in samples (mean of days −3 and 0) from the 3 diet groups were analyzed by using analysis of variance or Wilcoxon's rank sum scores. Differences between the end samples from the 3 diet groups were assessed by using either analysis of variance followed by Tukey's t test or Wilcoxon's rank sum scores followed by the Wilcoxon two-sample test.

A repeated-measures analysis of covariance was performed on the eligible data from all days from run-in to end or follow-up. Interactions with the run-in value (normalized to a mean of zero) and with diet, sex, and study section were all included in the model. If the time × diet interaction was significant by this analysis, Tukey's t test was performed to assess differences on each day.

Pearson correlation analyses were performed on run-in values and on the changes (difference between run-in and end values) caused by the intervention. All statistical analyses were performed by using the SAS statistical package version 6.12 (SAS Institute Inc, Cary, NC).

RESULTS

Participants and run-in measurements

All participants had stable weight during the trial, and overall changes in weight were <1.0 kg. Dropouts led to an underrepresentation of participants in the vitamin and mineral supplement group (n = 12) compared with the placebo group (n = 16) and the Fruveg group (n = 15). Age distributions and body mass index did not differ significantly between the groups and were unaffected by the dropouts.

The habitual dietary intakes of the subjects in the 3 groups are shown in **Table 5**; the intakes were calculated on the basis of a 4-d food record. There were no significant differences between the 3 groups. The intakes of β-carotene, vitamin C, and folate were higher in the subjects assigned to the Fruveg group than in the subjects who were assigned to the supplement and placebo groups, which reflects apparent but nonsignificant differences between the Fruveg group and the 2 other groups in the habitual intake of fruit and vegetables.

The values at run-in for all biomarkers are shown in the first 3 columns of **Table 6**. There were no significant overall differences between the 3 groups in these markers at run-in.

Several dietary exposure markers, including plasma vitamin C, serum lycopene, and serum β-carotene, confirmed the compliance of the participants. Plasma vitamin C and serum

TABLE 6
Antioxidant defense markers at run-in (habitual diet) and after the intervention in the 3 groups[1]

	Run-in (mean of days −3 and 0)			End (mean of days 24 and 25)		
Biomarker	Placebo group	Supplement group	Fruveg group	Placebo group	Supplement group	Fruveg group
Isoprostanes in urine (nmol/24 h)	10.3 ± 1.4	10.7 ± 1.7	9.8 ± 1.4	10.3 ± 1.2	10.3 ± 1.1	10.0 ± 1.4
Plasma MDA (pmol/mg protein)	40.3 ± 8.5	40.0 ± 10.6	37.7 ± 7.6	37.0 ± 5.7	36.0 ± 4.5	38.0 ± 6.5
Plasma carbonyls (relative intensity)	54.9 ± 7.4	53.1 ± 9.0	52.1 ± 4.3	52.8 ± 4.8	50.8 ± 6.1	52.7 ± 7.6
TEAC (μmol/L)	879 ± 101	920 ± 110	864 ± 100	876 ± 94	883 ± 88	889 ± 95
FRAP (μmol/L)	673 ± 154	706 ± 131	704 ± 1849	652 ± 179	707 ± 138	685 ± 158
SOD (U/g Hb)	950 ± 104	1009 ± 87	984 ± 158	981 ± 115	984 ± 104	993 ± 150
CAT (U/g Hb)	12.5 ± 2.0	13.7 ± 1.7	13.6 ± 2.4	13.3 ± 2.5	13.8 ± 2.3	13.0 ± 2.2
Gpx (U/g Hb)	116 ± 11	120 ± 20	126 ± 21	116 ± 16	119 ± 18	133 ± 22[2]
GR (U/g Hb)	11.5 ± 1.3	11.8 ± 1.5	10.8 ± 1.0	11.4 ± 1.9	11.7 ± 1.8	10.8 ± 0.9
GST (U/g Hb)	4.57 ± 1.01	4.30 ± 0.81	4.86 ± 1.63	5.47 ± 1.23	5.20 ± 0.88	5.74 ± 1.34

[1] All values are $\bar{x}$ ± SD. Samples were collected after the subjects had fasted for 12 h. Fruveg, fruit and vegetables; MDA, malondialdehyde; TEAC, plasma Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carbonic acid; Aldrich Chemical Co, Steinheim, Germany)-equivalent antioxidant capacity; FRAP, ferric-reducing capacity of plasma; SOD, superoxide dismutase; CAT, erythrocyte catalase; Gpx, erythrocyte glutathione peroxidase; GR, erythrocyte glutathione reductase; GST, whole-blood glutathione *S*-transferase.

[2] Significantly different from both the placebo group and the supplement group, $P < 0.05$ (Tukey's *t* test).

β-carotene decreased significantly with time in the placebo group, whereas lycopene decreased significantly in both the placebo and supplement groups because this compound was not part of the supplement (**Figure 2**). Although the content of β-carotene in the supplement was only 50% of that in the fruit and vegetables offered to the Fruveg group, the bioavailability of β-carotene in the supplement was much higher, which resulted in significantly higher serum concentrations of β-carotene in the supplement group than in the other 2 groups. For plasma ascorbate and folate, there were no significant differences between the Fruveg and supplement groups. For α-tocopherol (details on tocopherol and folate to be presented elsewhere), serum concentrations in the supplement group were significantly higher (15%) than those in the Fruveg group at the end of the intervention ($P = 0.03$).

Markers of oxidative damage

Plasma lipoprotein resistance increased significantly after the intervention in the Fruveg and supplement groups. The plasma lipoprotein resistance in the Fruveg group was also significantly higher than that in the supplement group at the end of the intervention (**Figure 3**). The overall oxidative damage to lipids showed no indication of changes in the general level of lipid oxidation as determined by urinary isoprostanes and plasma MDA (Table 6).

The formation of protein carbonyls at lysine residues (AAS) in total plasma proteins was also significantly affected by the treatments. In the Fruveg and supplement groups, there was a significant increase in the formation of lysine carbonyls at the end of the intervention, which indicates that some vitamin or mineral causes a specific prooxidant stress that affects plasma lysine sites. The time course of AAS formation in the placebo group shows that uncompensated omission of fruit and vegetables led to an immediate drop in this biomarker, which reached a nadir 9–16 d after this dietary change (**Figure 4**). After restoration to the habitual diet, plasma protein lysine carbonyls gradually increased and reached run-in values sometime between 1 and 4 wk after the end of the intervention.

Using a semiquantitative electrophoretic technique, we evaluated total carbonyls (including ketones) in 4 plasma proteins having a molar weight close to that of albumin. We observed no significant overall change in carbonyls in any of the proteins or in the sum of total carbonyls in all 4 proteins, although there was a trend toward an increase in the oxidation of 2 of the proteins after consumption of the Fruveg diet compared with consumption of the placebo diet (data not shown).

Plasma antioxidant capacity

None of the 4 markers of fasting plasma antioxidant capacity were significantly affected by the diets at the end of the intervention, as determined by analysis of variance (Table 6). The same was true for TEAC and FRAP measured postprandially (**Table 7**). Postprandial FRAP tended to increase in the Fruveg and supplement groups; however, this increase was not significant. By repeated-samples analyses of covariance, there was no overall change in TEAC with time, diet, or study section. FRAP was significantly affected by sex and was also affected by the run-in value, which was significantly lower (25%) in the women than in the men.

Oxygen consumption in plasma oxidized ex vivo was determined only at commencement and on the last day of the intervention. The dietary interventions did not significantly influence this marker.

Enzyme induction

Erythrocyte activities of superoxide dismutase, catalase, glutathione reductase, and GST were not significantly affected by the dietary interventions (Table 6). At the end of the intervention, Gpx activity was significantly higher in the Fruveg group than in the placebo and supplement groups. There was no significant difference between the run-in and the follow-up values. A repeated-samples analysis of covariance of the time period from run-in to follow-up 4 wk after the end of the intervention showed a significant decrease in Gpx activity in the Fruveg group compared with the other 2 groups ($P = 0.04$). Gpx activity values in the Fruveg group both at the end of the intervention and 1 wk later were significantly higher than the respective values in the placebo group (**Figure 5**).

GST activity increased with time during the intervention in all 4 study sections, but GST did not change significantly after the

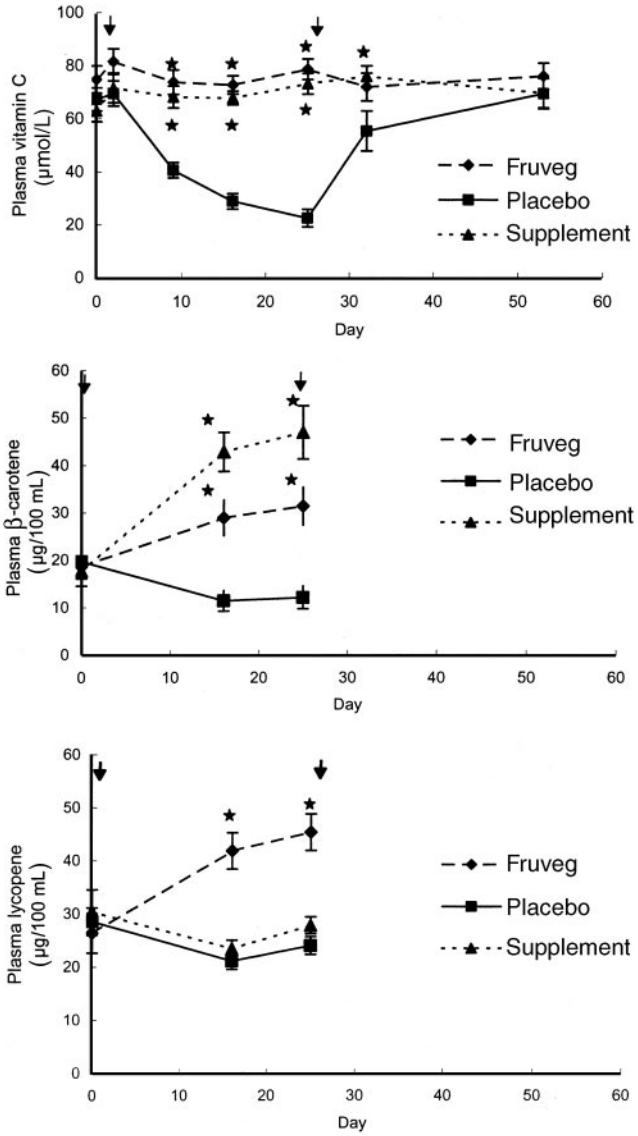

FIGURE 2. Mean (± SE) plasma concentrations of antioxidative nutrients before, during, and after dietary intervention in the placebo group (n = 15), the fruit and vegetables (Fruveg) group (n = 16), and the supplement group (n = 12). The start and the end of the intervention diet are marked with vertical arrows. *Significantly different from the placebo group, $P < 0.05$ (repeated-samples analysis of covariance followed by Tukey's t test).

end of the intervention when the subjects returned to their habitual diets. The run-in values also increased with increasing section number. Thus, there was a significant overall increase in GST over the course of the whole experiment from January to May. Subjects with high initial GST activity tended to have high activities throughout the trial and vice versa.

Interactions with other factors

A tendency toward a certain individual activity value was observed not only for GST but also for other markers, including AAS, glutathione reductase, superoxide dismutase, MDA, LDL-MDA, TEAC (including postprandial TEAC), reduced glutathione, and plasma vitamin C. This was shown by significant time × run-in interactions in repeated-measures analysis of covariance. Small but significant effects of time in the trial, independent of intervention group, were also observed for several markers, including GST, reduced glutathione, TEAC, MDA, and LDL-MDA. These effects probably reflect an overall effect of entering a trial and the change from a habitual diet to a defined diet.

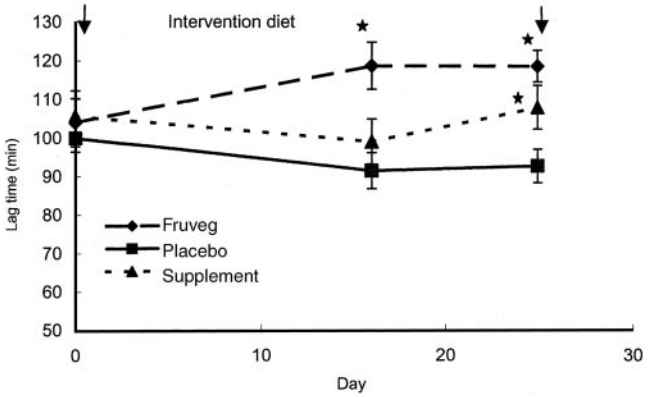

FIGURE 3. Mean (± SE) lipoprotein oxidation lag times in the 3 diet groups before and during dietary intervention in the placebo group (n = 15), the fruit and vegetables (Fruveg) group (n = 16), and the supplement group (n = 12). The start and the end of the intervention diet are marked with vertical arrows. *Significantly different from the placebo group, $P < 0.05$ (repeated-samples analysis of covariance followed by Tukey's t test).

Correlation analyses

Pearson correlation analysis of the markers indicated strong positive relations between plasma lipoprotein oxidation, TEAC, and FRAP in the run-in measurements ($r > 0.59$, $P < 0.0001$) and between LDL-MDA and both plasma lipoprotein oxidation ($r = 0.43$, $P = 0.03$) and radical-induced plasma oxygen consumption ($r = 0.49$, $P = 0.010$). The changes in TEAC and in FRAP during the intervention were correlated both postprandially and after fasting ($r > 0.80$, $P < 0.0001$), which indicates close relations between these 2 markers. Another cluster in the run-in values consisted of positive correlations between AAS and plasma vitamin C (**Figure 6**B). During the intervention, changes in AAS and in plasma vitamin C were also correlated, which indicates a strong relation between these markers (Figure 6A). A third cluster at run-in consisted of glutathione reductase, urinary isoprostanes, and radical-induced plasma oxygen consumption, all of which showed significant pair-wise correlations ($r > 0.41$, $P < 0.04$). Because none of these markers was affected by the dietary changes, further studies are necessary to confirm any real relation between these markers.

DISCUSSION

There is only limited evidence that antioxidants in fruit and vegetables influence oxidative stress or antioxidative defense in healthy subjects. In particular, the contribution of nonnutritive antioxidants to the prevention of oxidative damage is uncertain. The present biomarker-based, fully controlled human intervention study supports the hypothesis that nonnutritive factors, as well as nutritive ones, in fruit and vegetables may influence oxidative damage; however, the effect is not uniformly protective and depends on the molecular structure targeted by each biomarker.

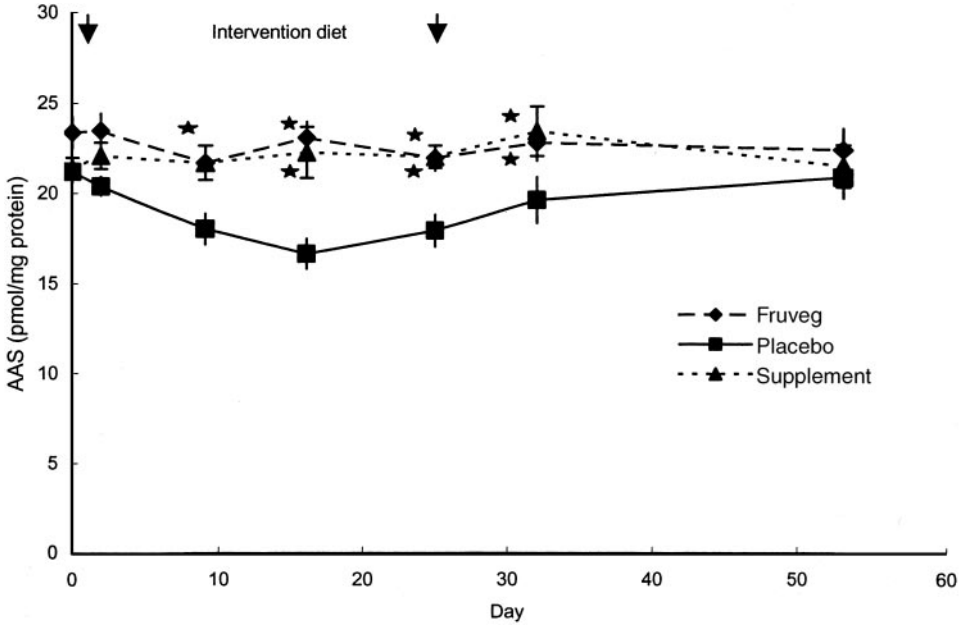

FIGURE 4. Mean (± SE) concentrations of amino adipic semialdehyde (AAS), a measure of protein oxidation, before, during, and after dietary intervention in the placebo group ($n = 15$), the fruit and vegetables (Fruveg) group ($n = 16$), and the supplement group ($n = 12$). The start and the end of the intervention diet are marked with vertical arrows. *Significantly different from the placebo group, $P < 0.05$ (repeated-samples analysis of covariance followed by Tukey's *t* test).

Markers of lipid oxidation in humans are thought to be important specifically for LDL oxidation or resistance and are therefore potentially important for prevention of atherosclerosis and ischemic heart disease (14). In the present study, we used 2 general markers of lipid oxidation, namely, 8-isoprostane $F_{2\alpha}$ in urine and MDA in plasma, and 2 markers specific for LDL oxidation, namely, direct determination of MDA in LDL and plasma lipoprotein resistance to oxidation ex vivo. The relation of the 2 former markers to dietary antioxidant intake is controversial, but in well-controlled dietary studies of fruit and vegetables, there were minimal effects on these markers, which is in accordance with our present observations (57, 58). In studies on specific antioxidants, neither tea polyphenols, vitamin C or E, nor isoflavonoids were found to significantly affect the excretion of 8-isoprostane $F_{2\alpha}$ in short-term human intervention studies (59–64). We used 2 other markers in the present study to assess lipid oxidation specifically in lipoproteins, namely, LDL-MDA and plasma lipoprotein resistance. Although the 2 markers were strongly and inversely correlated at baseline, only the latter marker was affected at the end of the intervention, possibly because of a lack of power for LDL-MDA. A significant and time-dependent increase in lipoprotein lag time occurred in the Fruveg and supplement groups. The increase in the Fruveg group was significantly higher than that in the supplement group, which indicates that additional factors, apart from the known vitamins and minerals, in fruit and vegetables affect lipoprotein oxidation. Several compounds, including vitamin E, vitamin C, and flavonoids (in trace amounts), have been shown to inhibit lipoprotein oxidation in human serum in vitro (48). In 3 studies without parallel control groups and with minimal control of the diets, lycopene-rich dietary interventions increased LDL-oxidation lag times (22, 34, 65). Plasma lycopene may therefore explain part of the additional effect of fruit and vegetable intervention on ex vivo lipoprotein oxidation. Other researchers have observed an increase in lipoprotein oxidation after α-tocopherol intervention (21). Because we observed minimal differences in tocopherols between the supplement and Fruveg groups during the intervention in the present study (data to be detailed elsewhere), α-tocopherol apparently did not cause the difference in ex vivo lipoprotein oxidation that was observed in the present study.

Assessment of protein oxidation is important in understanding the interactions between redox processes and functional changes.

TABLE 7
Markers of postprandial plasma antioxidant capacity at run-in (habitual diet) and after the intervention in the 3 groups[1]

	Run-in (day 0)			End (day 25)		
Biomarker	Placebo group	Supplement group	Fruveg group	Placebo group	Supplement group	Fruveg group
TEAC (μmol/L)	950 ± 170	900 ± 110	870 ± 170	880 ± 150	920 ± 140	870 ± 90
FRAP (μmol/L)	731 ± 236	777 ± 145	683 ± 181	652 ± 179	793 ± 123	685 ± 158
Plasma oxygen consumption (relative rate)	−68.3 ± 31.1	−57.8 ± 27.9	−65.8 ± 24.2	−68.6 ± 19.8	−56.7 ± 16.5	−57.6 ± 17.6

[1] All values are $\bar{x}$ ± SD. Fruveg, fruit and vegetables; TEAC, plasma Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carbonic acid; Aldrich Chemical Co, Steinheim, Germany)-equivalent antioxidant capacity; FRAP, ferric-reducing capacity of plasma. There were no significant differences between groups or over time.

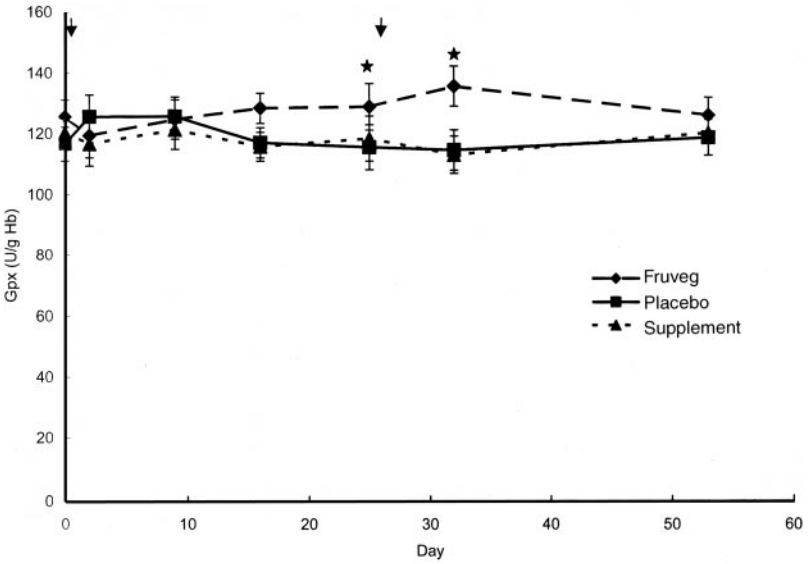

FIGURE 5. Mean (± SE) erythrocyte glutathione peroxidase (Gpx) activity before, during, and after dietary intervention in the placebo group ($n = 15$), the fruit and vegetables (Fruveg) group ($n = 16$), and the supplement group ($n = 12$). The start and the end of the intervention diet are marked with vertical arrows. *Significantly different from the placebo group, $P < 0.05$ (repeated-samples analysis of covariance followed by Tukey's t test). Hb, hemoglobin.

In LDL, the lysine-rich apolipoprotein B-100 is important for recognition by receptors. Oxidation at lysine residues seems to be important for interaction with the scavenger receptor (66, 67). We developed AAS some years ago as a marker of plasma protein carbonyls specifically at lysine residues (49). AAS has previously been observed to increase with air pollution from traffic in bus drivers, who are known to have an excess rate of heart disease (68). Paradoxically, AAS also increases with a vitamin C–rich intervention and decreases when ascorbate is omitted from the intervention diet (32, 33, 36). This is in accordance with our present finding of persistently high concentrations of AAS in the subjects in the Fruveg and supplement groups; the concentrations in those groups were significantly higher than those in the placebo group during most of the intervention period. The time course is almost identical to that for the change in plasma vitamin C. Positive correlations between AAS and plasma vitamin C were reported previously in a group of Dutch volunteers (69). The present finding of a completely similar time course for AAS and plasma vitamin C corroborates the conclusion that vitamin C acts as a prooxidant toward protein lysine sites, at least in the plasma compartment. In support of this conclusion, the run-in values and changes from run-in to the end of the intervention for AAS correlate strongly with those for vitamin C.

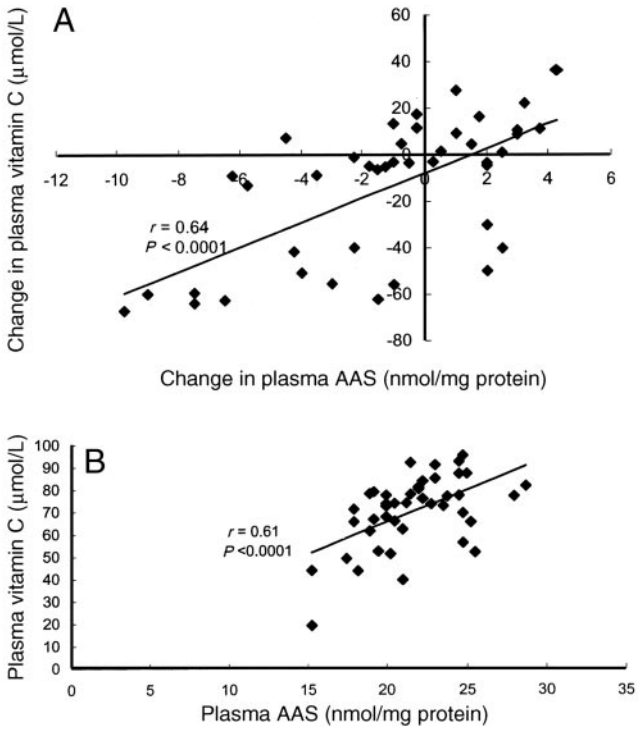

FIGURE 6. Correlations between changes in plasma vitamin C concentrations and changes in plasma concentrations of amino adipic semialdehyde (AAS), a measure of protein oxidation, from the habitual diet (mean of days 0, 2, and 53) to the end of the intervention period and between plasma vitamin C concentrations and plasma AAS concentrations during the habitual diet. Each point represents a data set (plasma AAS and plasma vitamin C) from one of the subjects. The line in each panel is a least-squares regression line.

Using a semiquantitative electrophoretic technique, we found no significant differences within or between the 3 diet groups in total carbonyls in 4 unknown plasma proteins having a molar weight close to that of albumin. Other researchers have reported a decrease in plasma globulin carbonyls after vitamin C intervention (70), which is in contrast with our findings. Differences in methodology and study design are likely to have caused these differences.

The plasma antioxidant capacity measures TEAC and FRAP were very strongly correlated with each other at run-in, and there was no major difference between postprandial and fasting measures. FRAP is known to be predominantly a measure of plasma uric acid (71), and therefore the significant sex differences in this marker that we observed in the present study and those observed by other researchers (72) are not surprising. Postprandial effects on FRAP and TEAC after intake of plant foods are controversial (69, 73, 74). In fasting samples, no effects of polyphenols on FRAP or TEAC were reported (32–33, 36). In one of these studies (32), we noted a postprandial effect on ex vivo radical-

induced plasma oxygen consumption, but such an effect was not apparent in the present study. Fruit and vegetables apparently do not affect plasma antioxidant capacity measures, whereas food items with very high amounts of specific plant phenols may have postprandial effects.

Gpx activity increased with the Fruveg intervention, but not with the supplement intervention, which indicates that nonnutrients in fruit and vegetables may have caused this effect. The increase had a relatively late onset and continued for a week after the return to the habitual diet, but Gpx activity was back to run-in values after 4 wk of follow-up. We previously observed that Gpx activity increases after intervention with berries after much shorter intervention times, which indicates that extra-genetic regulation may take place for this enzyme in erythrocytes (32, 33). The activity of GST, a phase II enzyme, was unaltered in erythrocytes by the interventions in the present study. The isoenzyme GST P1 contributes most of the activity determined by the substrate used in the present study (75). Similar findings were reported from a recent pilot study on the expression of the GST-P isoenzyme in lymphocytes in 6 subjects undergoing a 3-wk vegetable intervention (76). We found that erythrocyte GST activity increased throughout the present study, both within each of the 4 sections and with increasing section number (ie, there was a persistent increase from January to May). We previously published similar results for Ogg1 expression (77), which seems to be influenced by increasing exposure to sunlight. However, changes in GST and Ogg1 expression did not correlate at the individual level. It is therefore likely that other seasonal factors are behind the increase in GST activity.

The antioxidant hypothesis of chronic disease prevention does not explain the complex interactions that were observed in the present study and in other studies between various antioxidants and molecular targets in cells and body fluids. For instance, the different biomarkers that assess oxidative damage in lipids, proteins, and DNA are generally not well correlated. Because the biomarkers used in the present study are reproducible in the sense that individuals were found to maintain their own characteristic values throughout the measurements, the most apparent explanation is that redox processes leading to oxidative damage are tightly regulated and localized to specific molecular targets.

The overall picture from the present study is that fruit and vegetables at the recommended dietary intake have an effect only on markers of oxidative damage to plasma proteins and lipoproteins and on enzymatic defense. The latter 2 effects seem to be effected mainly by nonnutrients.

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BS, LOD, SL, AP, and SER planned the study. Recruitment of volunteers, planning, scheduling and checking volunteers, and overseeing kitchen personnel and the handling of all specimens, including collection, transfer, and storage, were performed by AP. Analytic responsibilities were as follows: antioxidant enzymes, TEAC, and FRAP (LOD and GRH); lipoprotein oxidation (AH); urinary isoprostanes (SB); protein lysine oxidation and MDA (MH and LOD); plasma and dietary vitamin C (MK); GST (VB); carotenoids in plasma (JJMC); protein carbonyls by electrophoresis (JS and MH); oxygen consumption assay (LS). Collection of raw data and final code matching were performed by AP. Statistical analyses were done by LOD and AP. The draft manuscript was prepared by LOD, and all coauthors participated in critically revising the manuscript for important intellectual content. None of the authors had any financial or personal interests, including advisory board affiliations, in any company or organization sponsoring the research.

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