Protection of Multiple Antioxidants against Heme Protein Oxidation and Lipid Peroxidation Induced by $CBrCl_3$ in Liver, Lung, Kidney, Heart, and Spleen

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Protection of multiple antioxidants against heme protein oxidation and lipid peroxidation induced by bromotrichloromethane ($CBrCl_3$) was studied in a tissue homogenate system. Rats were fed a basal diet (vitamin E and selenium deficient) supplemented with different kinds and amounts of antioxidants. Diet 1 was supplemented with vitamin E and selenium (control diet). Diet 2 was supplemented with vitamin E, selenium, and the water-soluble/less fat-soluble antioxidants coenzyme Q_0, trolox c, and acetylcysteine. Diet 3 was supplemented with vitamin E, selenium, and both water- and fat-soluble antioxidants trolox c, coenzyme Q_0, acetylcysteine, β-carotene, ascorbic acid 6-palmitate, coenzyme Q_{10}, and canthaxanthin. (+)-Catechin was added to diets 2 and 3 as a representative flavonoid. In general, lower concentrations of thiobarbituric acid reactive substances and oxidized heme proteins were found in tissues from rats fed diets 2 and 3 than from rats fed diet 1, suggesting that increased quantity and diversity of antioxidants in diets significantly strengthen protection against oxidative damage to tissues.

Keywords: *Heme proteins oxidation; lipid peroxidation; multiple antioxidants; liver; lung; kidney; heart; spleen; bromotrichloromethane*

INTRODUCTION

Recent studies have demonstrated that intake of dietary antioxidants has beneficial effects on prevention of human diseases. For instance, a high dose of vitamin E was able to reduce the risk of coronary disease by more than 50% in both men and women (Rimm *et al*., 1993; Stampfer *et al*., 1993). Since dietary antioxidants from food and food supplements provide potential means for prevention of certain human diseases, increased antioxidant protection against oxidative damage of tissue is an important subject to study.

Synergistic effects among individual antioxidants have been studied *in vitro*, and experimental results have shown that the protective effect of individual antioxidants can be augmented in the presence of other antioxidants (Niki *et al*., 1982; Niki, 1987, 1991; Motoyama, 1989). If synergistic effects among antioxidants are able to increase the effectiveness of individual antioxidants against oxidative damage, it would be expected that increased diversity of antioxidants in animal diets would provide better protection against oxidative damage. Thus, it is important to test this concept in animal dietary experiments.

Numerous chemicals can initiate oxidative damage processes in animal tissues. For example, halogenated hydrocarbons found in contaminated drinking water, food, and polluted air can be potent inducers for free radical chain reactions (Recknagel *et al*., 1977; Reynolds and Moslen, 1980; *Chemical & Engineering News*, 1984; Hidalgo *et al*., 1990). The mechanisms of CCl_4 and $CBrCl_3$ (the prototypes of halogenated hydrocarbons) in the initiations of oxidative damage processes in biological systems have been studied. The highly reactive oxygen species, such as trichloromethyl and trichloromethylperoxyl radicals, generated during the metabolism of CCl_4 by cytochrome P450 monoxygenase systems can react with various amino acids and unsaturated lipid rapidly, thus causing oxidative denaturation of proteins and lipid peroxidation (Noguchi *et al*., 1982). The mechanism of $CBrCl_3$-induced oxidative damage in tissues is believed to be similar to that of CCl_4-induced oxidative damage (Fraga *et al*., 1989). Dietary antioxidants, such as vitamin E and β-carotene, act as free radical scavengers and thus can effectively intercept the reactive oxygen species generated from the metabolism of xenobiotics/chemicals. Protection by dietary antioxidants against halogenated hydrocarbon induced oxidative damage, therefore, is an appropriate model to study the effects of antioxidants on the chemically induced oxidative damage to tissues.

In recent studies we have demonstrated that increased diversity of dietary antioxidants significantly strengthened the capabilities of liver to counteract oxidative damage to heme proteins (Chen and Tappel, 1994). In the present study we further investigated the protection of multiple antioxidants against oxidative damage to liver, lung, kidney, heart, and spleen. Rats were fed a vitamin E and selenium deficient diet (basal diet) supplemented with vitamin E and selenium (diet 1), supplemented with vitamin E, selenium, and water-soluble/less fat-soluble antioxidants (trolox c, coenzyme Q_0, and acetylcysteine) (diet 2), or supplemented with vitamin E, selenium, and both water soluble and fat soluble antioxidants (trolox c, coenzyme Q_0, acetylcysteine β-carotene, ascorbic acid 6-palmitate, coenzyme Q_{10}, canthaxanthin) (diet 3). (+)-Catechin was added to diets 2 and 3 as a representative flavonoid. Three advancements were made in this study. Bromotrichloromethane ($CBrCl_3$) is present in the environment and is used in scientific studies as a halogenated hydrocarbon that is a powerful initiator of oxidative damage (Sano and Tappel, 1990). The trichloromethyl radical from $CBrCl_3$ will abstract hydrogen, inducing lipid peroxidation in tissue slices as is shown by the produc-

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0021-8561/96/1444-0854$12.00/0

Table 1. Addition of Antioxygenic Nutrients to Basal Diet

antioxidant	amt of antioxidant added (mg/kg of diet)		
	diet 1	diet 2	diet 3
vitamin E[a]	25	25	25
selenium[b]	0.3	0.3	0.3
β-carotene	0	0	45
coenzyme Q_{10}	0	0	30
ascorbic acid 6-palmitate	0	0	100
canthaxanthin	0	0	45
trolox c	0	50	50
acetylcysteine	0	200	200
coenzyme Q_0	0	100	100
(+)-catechin	0	100	100

[a] (+)-α-Tocopherol acid succinate. [b] As sodium selenite.

tion of TBARS. Intraperitoneal injection of $CBrCl_3$ without incubation also induces the formation of oxidized heme proteins in liver, lung, kidney, spleen, and heart (Chen and Tappel, 1995a). In this investigation $CBrCl_3$ was injected into animals intraperitoneally. With this technique $CBrCl_3$ was transported to the target tissues by the circulation of blood. Second, in addition to liver, protection by multiple antioxidants against oxidative damage to lung, kidney, heart, and spleen was also investigated. Measurement of both oxidized heme proteins and lipid peroxidation induced by $CBrCl_3$ was measured to determine the effectiveness of antioxidants in protection of tissues against oxidative challenge.

MATERIALS AND METHODS

Chemicals. The antioxidants and chemicals used in this study were α-tocopherol acid succinate (1210 IU/g), sodium selenite (Alfa Inorganics, Beverly, MA), trolox c (Aldrich Chemical Co., Milwaukee, WI), coenzyme Q_0, coenzyme Q_{10}, (+)-catechin, dimethyl sulfoxide, *trans*-β-carotene, L-ascorbic acid 6-palmitate, acetylcysteine (Sigma Chemical Co., St. Louis, MO), canthaxanthin (Roche Vitamin and Fine Chemicals, Nutley, NJ), and $CBrCl_3$ (Eastman Kodak Co., Rochester, NY).

Animals and Diets. Male Sprague-Dawley rats (Bantin & Kingman, Fremont, CA) weighing 40−60 g were fed experimental diets. The basal diet was a vitamin E and selenium deficient diet with 10% tocopherol stripped corn oil (Teklad Test Diet TD 77068, mineral mix 170911, Teklad Test Diets, Madison, WI). Animals were housed according to NIH guidelines and had free access to deionized water and food. Dietary treatment had no effect on weight gain of the animals. Rats were fed either basal diet supplemented with vitamin E and selenium (diet 1); basal diet supplemented with vitamin E, selenium, trolox c, coenzyme Q_0, and acetylcysteine, (diet 2); or basal diet supplemented with vitamin E, selenium, trolox c, coenzyme Q_0, acetylcysteine, β-carotene, ascorbic acid 6-palmitate, coenzyme Q_{10}, and canthaxanthin (diet 3). (+)-Catechin was added to diets 2 and 3 as a representative flavonoid. The protocol for the dietary study is shown in Table 1. The rats were on the experimental diets and distilled water for 6 weeks.

Preparation of Tissue Homogenates. After the respective dietary treatments, rats were injected intraperitoneally with $CBrCl_3$ (0.05 mmol/100 g of body weight in mineral oil) or mineral oil (control) and sacrificed 40 min after the injection. The rats were decapitated, and liver, lung, kidney, heart, and spleen were immediately dissected and immersed in ice-cold Krebs−Ringer phosphate (KRP) buffer (pH 7.4). Organs were blotted with filter papers and stored at −22 °C. Frozen organs were cut into 0.5-cm^3 cubes by a sharp surgical knife, and homogenates were prepared by homogenizing 1 g of tissue with 9 mL of oxygenated KRP buffer containing glucose (10 mmol, pH 7.4). A motor-driven tissue homogenizer was used.

Oxidation of Heme Proteins in Tissue Homogenates. Oxidative damage of heme proteins induced by $CBrCl_3$ in tissue homogenates of liver, lung, kidney, heart, and spleen was conducted in the following manner: tissue homogenates (1.5 mL) were transferred to a 10-mL glass serum bottle and covered with a layer of parafilm. The homogenates were incubated in a gyrotory water bath shaker (New Brunswick Scientific Co., Inc., New Brunswick, NJ) at 37 °C with continuous shaking (180 cycles/min).

Spectrophotometric Measurement of Heme Proteins from Tissue Homogenates. After incubation, the absorbance spectra of tissue homogenates were obtained with a Beckman DU-50 spectrophotometer (Beckman Instruments, Inc., Fullerton, CA). Tissue homogenates (0.6 mL) were transferred to a microcuvette with a light path of 10 mm and mixed with 0.6 mL of glycerol. Four layers of parafilm representing turbidity were used as background to subtract some of the absorbance caused by turbidity inherent in tissue homogenates. The sample was scanned from 500 to 640 nm, and absorbance vs wavelength at 5-nm intervals was automatically recorded by a scan program in the spectrophotometer.

Analysis of Absorbance Spectra of Heme Proteins of Tissue Homogenates with HPSAP. HPSAP is a spreadsheet program written with Lotus 1-2-3 (Lotus Development Corp., Cambridge, MA) that contains visible spectra of individual heme proteins from 500 to 640 nm. HPSAP is based on the knowledge that the absorbance spectrum of a mixture of heme proteins is the sum of the spectra of the individual heme proteins including any contribution from turbidity of the biological samples. Quantitation of individual heme proteins is achieved by matching the calculated spectrum with the experimental spectrum through successive approximations. The determination of heme proteins with HPSAP can be highly accurate since the visible absorbance of heme proteins closely obeys Beer's law. Any interaction between individual heme proteins, such as redox reactions, will result in other measurable heme proteins. The details of the development and application of HPSAP in a liver homogenate model system are described in our previous studies (Chen *et al.*, 1993; Chen and Tappel, 1993).

Oxidation of Homogenates and Measurement of TBARS. Oxidation of tissue homogenates and measurements of TBARS followed the methods described by Zalkin and Tappel (1960) with some modifications. Two milliliters of homogenate was transferred to a 10-mL glass serum bottle containing 3 mL of oxygenated 0.9% NaCl. The homogenates were incubated in the water bath shaker at 37 °C with continuous shaking (180 cycles/min). After incubation, the bottles were cooled with dry ice. Homogenates (2.5 mL) were transferred to a 10-mL centrifuge tube containing 2.5 mL of 10% TCA. The homogenates were centrifuged at 3000 rpm for 2 min at 4 °C. After centrifugation, the method proceeded as described previously. TBARS were expressed as MDA equivalents per gram of tissue.

Statistical Analysis. The statistical package SAS (SAS Institute Inc., Cary, NC) was used to analyze all data. When significant F values were obtained using ANOVA, Duncan's lsd procedure was used to determine significant difference ($P \leq 0.05$) between treatment means. Results were expressed as means ± standard deviation.

RESULTS

Figure 1 presents the oxidative damage to heme proteins and the protection provided by diets supplemented with either vitamin E and selenium or vitamin E and selenium plus other antioxidants in liver, lung, kidney, heart, and spleen. Formation of oxidized heme proteins (OHP) at time 0 can be related to *in vivo* oxidative damage caused by intraperitoneal injection of $CBrCl_3$. The statistical evaluation of antioxidant protective effects at time 0 is included in ANOVA analysis, and the overall results are shown in Figure 1. Quantities of *in vivo* oxidized heme proteins in kidney were much greater than those in liver, lung, heart, and

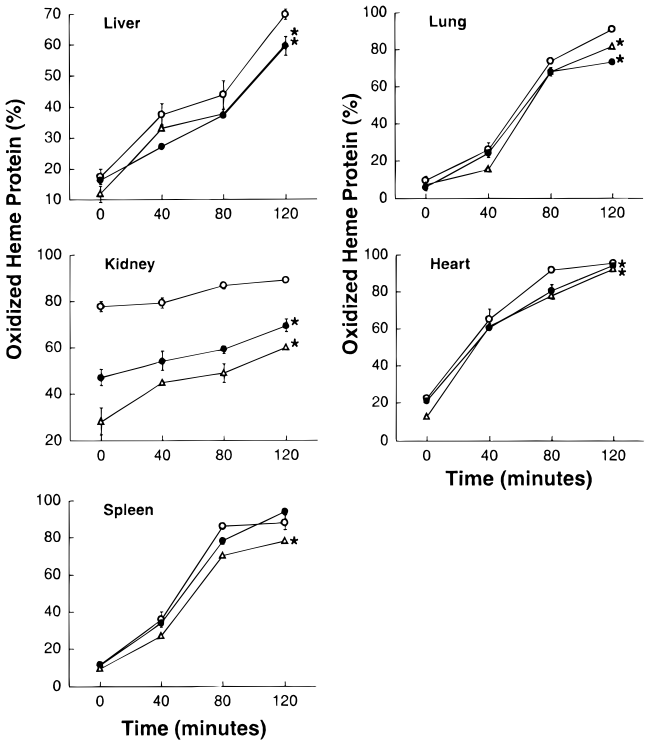

Figure 1. Oxidative damage to liver, lung, kidney, heart, and spleen measured by formation of OHP in tissue homogenates: (○) diet 1; (●) diet 2; (△) diet 3. Rats were injected intraperitoneally with $CBrCl_3$ (0.05 mmol/100 g of body weight in mineral oil) and sacrificed 40 min after the injection. Tissue homogenates were incubated at 37 °C. The values are expressed as mean ± SD for three tissue homogenate measurements. Values with asterisks indicate that the mean is significantly smaller than the mean of diet 1.

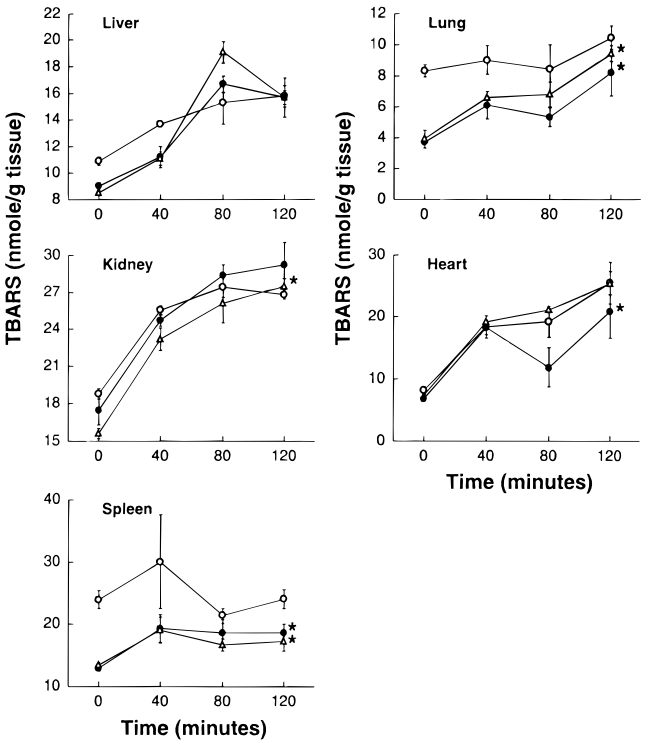

Figure 2. Oxidative damage to liver, lung, kidney, heart, and spleen measured by formation of TBARS in tissue homogenates: (○) diet 1; (●) diet 2; (△) diet 3. Rats were injected intraperitoneally with $CBrCl_3$ (0.05 mmol/100 g of body weight in mineral oil) and sacrificed 40 min after the injection. Tissue homogenates were incubated at 37 °C. The values are expressed as mean ± SD for four tissue homogenate measurements. Values with asterisks indicate that the mean is significantly smaller than the mean of diet 1.

spleen, suggesting that kidney may be the major target tissue for $CBrCl_3$-induced oxidative damage. Under *in vivo* situations, feeding rats with diet 3, containing both water and lipid soluble antioxidants, significantly lowered the formation of OHP in kidney, heart, and liver. Diet 2, containing mainly water-soluble antioxidants, protected kidney well (but less effectively than diet 3) against heme protein oxidation but failed to protect liver and heart in contrast to diet 3. Both diets 2 and 3 appeared to have no protection against oxidative damage to heme protein in lung and spleen. In general, during incubation diets containing vitamin E and selenium plus either additional water-soluble or water and fat-soluble antioxidants (diets 2 and 3) provided better protection against heme protein oxidation than the diet containing only vitamin E and selenium (diet 1). Both diets 2 and 3 appeared to be effective in reducing oxidative damage to heme proteins in kidney. After incubation for 40 min, approximately 80% of the heme proteins had been converted into OHP in kidney from rats fed diet 1. Approximately 55% and 45% of heme proteins had been converted to OHP in kidneys from rats fed diets 2 and 3, respectively, after the 40-min incubation. In spleen, only diet 3 significantly strengthened the protection of vitamin E and selenium.

Figure 2 shows formation of TBARS from lipid peroxidation induced by $CBrCl_3$ and the augmented protective effect of vitamin E and selenium by the addition of multiple dietary antioxidants. Formation of TBARS at time 0 can result from *in vivo* oxidation due to intraperitoneal injection of $CBrCl_3$. Statistical evaluation of antioxidant protective effects at time 0 is included in ANOVA analysis, and the overall results are shown in Figure 2. Concentrations of TBARS in kidney were higher than those in liver, lung, and heart. This observation is consistent with $CBrCl_3$-induced oxidative damage of protein in these organs and with kidney as the primary target organ for $CBrCl_3$-induced lipid peroxidation *in vivo.* Under *in vivo* conditions, both diets 2 and 3 showed significant lowering of the formation of TBARS in liver, lung, kidney, and spleen. Diets 2 and 3 appeared to have no protection against $CBrCl_3$-induced lipid peroxidation in heart. Interestingly, diet 2 performed as well as diet 3 in protecting specific organs against lipid peroxidation, suggesting that the quantities and the combinations of antioxidants in diet 2 may have achieved the maximum protection at least *in vivo.* During incubation diets 2 and 3 significantly reduced lipid peroxidation in lung and spleen. For example, after 40 min of incubation, the concentration of TBARS in spleen from rats fed diet 1 was approximately 30 nmol/g of tissue. Under the same experimental conditions, the concentration of TBARS was approximately 18 nmol/g of tissue from rats fed diet 2 or 3. Diets 2 and 3 also protected liver and lung from lipid peroxidation with shorter incubation time (40 min). Diet 3 provided better protection than either diet 1 or 2 in kidney against lipid peroxidation, while diet 2 significantly decreased the formation of TBARS in heart following the longest incubation times.

DISCUSSION

A recent study has indicated that a diet supplemented with vitamin E and selenium provided significant protection against heme protein oxidation and lipid

peroxidation to liver in contrast to a vitamin E and selenium deficient diet (Chen *et al*., 1993).

Protection by multiple dietary antioxidants against oxidative damage to liver has been reported and discussed in our previous studies (Chen and Tappel, 1994). Additional analysis of *in vitro tert*-butyl hydroperoxide or iron induced oxidative damage to heme proteins of rat tissue homogentates showed the greatest protection by the diet containing both fat-soluble antioxidants (vitamin E, β-carotene, coenzyme Q_{10}, and ascorbic acid palmitate) and water-soluble antioxidants [selenium, trolox C, acetylcysteine, coenzyme Q_0, and (+)-catechin] (Chen and Tappel, 1995b). It is important to further investigate how protection to other tissues can be augmented through the addition of multiple dietary antioxidants. Protection by vitamin E and selenium can be strengthened, at least in theory, by increasing the amounts of vitamin E and selenium. The effectiveness of antioxidants is normally proportional to their concentrations. This approach, however, could encounter potential problems. A high dose of selenium may cause toxic effects (Parizek, 1987). Vitamin E is not effective in intercepting a free radical chain reaction in the aqueous phase. Lung, kidney, heart, and spleen are not major storage sites for vitamin E due to their lower absorption of vitamin E. Concentrations of α-tocopherol in heart, kidney, spleen, and lung were 0.9, 5.2, 7.3 and 18.6 nmol/g of tissue, respectively, while the concentration of α-tocopherol in liver was 35.3 nmol/g of tissue after rats were fed supplementary vitamin E (Huang and Shaw, 1994). It is clear, therefore, that increasing dietary vitamin E can have some limitations in protecting heart, kidney, spleen, and lung effectively against oxidative damage. These potential problems led us to explore the efficaciousness of increasing the diversity of dietary antioxidants to strengthen the antioxidant protection by vitamin E and selenium. Acetylcysteine, a precursor in the biosynthesis of glutathione, may enhance the effectiveness of selenium since the activity of selenium-dependent glutathione peroxidase is also dependent upon the concentration of glutathione and sulfhydryl compounds. Addition of water-soluble antioxidants to diets may improve defense systems in the aqueous phase against oxygen free radicals and free radical chain reactions. Synergistic interactions between vitamin E and other antioxidants can improve the effectiveness of vitamin E (Tappel, 1993). Finally, lung, kidney, heart, and spleen may absorb other fat-soluble antioxidants better than vitamin E.

The present study indicates that diets containing highly diversified antioxidants can effectively protect kidney, lung, and spleen. As shown in Figure 2, diets 2 and 3 significantly reduced the *in vivo* (0 incubation time) formation of TBARS in lung and spleen. Formation of OHP in kidney *in vivo* was greatly reduced by feeding rats diets 2 and 3. Since lung, kidney, and spleen have lower tissue concentrations of α-tocopherol, the improvement of protection against oxidative damage in those tissues was apparently due to the additional antioxidants. Perhaps lung, kidney, and spleen absorb the other antioxidants better than vitamin E. Since the tissue concentration of vitamin E in heart is low, the less severe *in vivo* oxidative damage to heme proteins in heart compared to kidney and the less severe *in vivo* lipid oxidation in heart compared to kidney and spleen may be due to the lower concentration of $CBrCl_3$ or the lack of activation of $CBrCl_3$ in heart.

In this study, trolox c, coenzyme Q_0, acetylcysteine, β-carotene, ascorbic acid 6-palmitate, coenzyme Q_{10}, canthaxanthin, and (+)-catechin were incorporated into diets with vitamin E and selenium. Diets enriched with multiple antioxidants (diets 2 and 3) contained additional water-soluble or water- and fat-soluble antioxidants and thus could strengthen protection for both cellular membranes and the cytosol against oxidative challenge. Since most of the antioxidants used in this study are widely distributed in foods such as fruits and vegetables, the results of this investigation have practical significance.

In general, diets enriched with multiple antioxidants (diets 2 and 3) provided better protection against both heme protein oxidation and lipid peroxidation than the diet supplemented only with vitamin E and selenium (diet 1). There are three primary reasons for the superior performance of diets 2 and 3. First, besides vitamin E and selenium, almost all additional antioxidants in both diets 2 and 3 have individually shown potent antioxidant properties in either *in vitro* or *in vivo* systems (Niki, 1991; Burton and Ingold, 1989; Beyer, 1990; Krinsky, 1989). Combinations of those antioxidants and including dietary vitamin E and selenium would be expected to protect animals better than the diet containing only vitamin E and selenium. Second, as shown in Table 1, the total quantity of antioxidants in both diets 2 and 3 is significantly greater than in diet 1. The ratio of antioxidants vs oxidizing components in tissues from rats fed diets 2 and 3 would thus be expected to be higher than in rats fed diet 1. As a result, rats fed diets 2 and 3 were less vulnerable to oxidative challenge. Third, synergistic interactions of the antioxidants may also play a role in the superior protection provided by both diets 2 and 3. Studies have demonstrated that synergistic interaction among individual antioxidants may strengthen the protection provided by individual antioxidants. For example, vitamin C, the well-known water-soluble antioxidant, can be a synergist in conjunction with vitamin E (Niki, 1987, 1991). Experiments have also indicated that acetylcysteine could act as a reducing agent in the regeneration of vitamin E by interacting with α-tocopherol radicals (Niki *et al*., 1982; Motoyama *et al*., 1989). In this study, the antioxidant defense system, in some cases, contained as many as 10 dietary antioxidants (diet 3); interactions among antioxidants can be complicated. The mechanisms mentioned above can only partly explain the observed synergistic effect caused by the multiple antioxidants. Therefore, more detailed studies on the interactions among multiple antioxidants are needed to elucidate the fundamental chemical processes whereby multiple antioxidants protect tissues against oxidative damage.

The present studies suggest that if the synergism of multiple antioxidants is applicable *in vivo*, then increased diversity of antioxygenic nutrients in diets should improve the protective effect of vitamin E and selenium against heme protein oxidation and lipid perioxidation. Our results demonstrate that increased diversity of antioxidants in diets may be an important approach to improve the protection by individual antioxidants.

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Received for review August 22, 1995. Revised manuscript received December 28, 1995. Accepted January 10, 1996.[⊗]

JF950562F

[⊗] Abstract published in *Advance ACS Abstracts,* February 15, 1996.