Protection by Multiple Antioxidants Against Lipid Peroxidation in Rat Liver Homogenate

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ABSTRACT: The purpose of this study was to test the hypothesis that multiple antioxygenic nutrients provide increased protection against lipid peroxidative damage to rat liver. Rats were fed diets (i) deficient in vitamin E and selenium (Diet 1), (ii) supplemented with vitamin E and selenium (Diet 2), (iii) supplemented with (ii) and in addition trolox C, N-acetylcysteine, coenzyme Q10, and (+)-catechin (Diet 3), or (iv) supplemented with (iii) and in addition β-carotene, ascorbic acid palmitate, canthaxanthin, and coenzyme Q10 (Diet 4). Liver homogenates were obtained from three rats fed each of the diets for six weeks and were incubated at 37°C up to two hours with and without exogenous tertiary-butyl hydroperoxide (TBHP) or Cu2+. Lipid peroxidation was determined by measurement of thiobarbituric acid-reactive substances (TBARS), and this protection was augmented by Diet 4. Diets 2, 3, and 4 were protective against mild oxidation induced by TBHP or Cu2+. During incubations with exogenous TBHP and Cu2+, there were only small differences between diets supplemented with antioxidants in inhibition of lipid peroxidation, indicating that diets supplemented with vitamin E and selenium (Diet 2) may have provided the maximal protection for liver. The possible mechanisms of protection provided by multiple antioxidants in diets were discussed. Protection by multiple antioxidants against lipid peroxidation may translate to prevention of peroxidative damage to human tissue, a factor in human disease.


Lipid peroxidation, the major free radical reaction in cellular membranes, can cause serious oxidative damage to animal cells and tissues. Reactive oxygen species generated from lipid peroxidation rapidly interact with proteins, enzymes, and DNA molecules. Consequences of such interactions are involved in the development of various disease states such as atherosclerosis, cardiovascular disease, degenerative diseases, and cancer (1,2).

Vitamin E, ascorbic acid, thiol compounds, carotenoids, coenzyme Q, and flavonoids are potent antioxidants that can effectively inhibit lipid peroxidation and thus protect tissues against oxidative damage (3–5). Supplementary dietary antioxidants have shown beneficial effects in prevention of certain diseases. For example, some epidemiological studies have shown that dietary intake of high doses of vitamin E is associated with a reduced risk of heart disease (6).

If individual antioxidants can effectively inhibit lipid peroxidation both in vitro and in vivo as suggested by the above studies, it would be expected that a system containing multiple antioxidants could be more effective in protecting tissues against oxidative damage. First, multiple antioxidants in a system would provide complementary antioxygenic functions. A system containing both fat-soluble and water-soluble antioxidants will be more effective than a system containing only fat-soluble or water-soluble antioxidants because the former is capable of intercepting reactive oxygen species from both aqueous and lipid phases (3). Second, synergistic interaction could strengthen the functions of individual antioxidants. The antioxygenic functions of vitamin E can be augmented when ascorbic acid is present due to the participation of ascorbic acid in the regeneration of vitamin E (7). Third, addition of multiple antioxidants to diets would increase the total quantity of antioxidants. Recently, there have been studies of the effects of multiple antioxidants in cellular defense against oxidative damage (8–10).

The purpose of this study was to test the concept that diets containing multiple antioxidants would be more effective in protection of liver against lipid peroxidation. The diets were supplemented with vitamin E, trolox C (the water-soluble vitamin E analog), selenium, a thiol compound, carotenoids, coenzyme Q, ascorbic acid palmitate, and (+)-catechin, a flavonoid. There are three primary reasons for choosing these antioxidants. These antioxidants have shown strong protection against lipid peroxidation at least in in vitro systems. In addition, most of these antioxidants are present in human foods so that the results from this study would be applicable to people. Finally, effects of combinations of these dietary antioxidants have not been studied thoroughly. In this study, rats were fed either an antioxidant-deficient diet or diets containing multiple antioxidants for six weeks. To test the effectiveness of diets supplemented with antioxidants in protection against lipid peroxidation, liver homogenates were incubated at 37°C with and without the addition of the exogenous prooxidants tertiary-butyl hydroperoxide (TBHP) and Cu2+. Lipids, Vol. 31, no. 1 (1996).
TBHP is a representative toxic hydroperoxide, a class of compounds constantly formed in small amounts in normal metabolism. Cu²⁺ is a trace element which can initiate lipid peroxidation in vivo. Lipid peroxidation was measured as thiobarbituric acid-reactive substances (TBARS). The results from spontaneous lipid peroxidation provided information on the protection by multiple antioxidants against oxidative damage under conditions close to the in vivo situation. The results from lipid peroxidation induced by TBHP or Cu²⁺ showed the degree of effectiveness of multiple antioxidants in inhibition of lipid peroxidation initiated by specific prooxidants.

MATERIALS AND METHODS

Chemicals. The antioxidants and chemicals used in this study were (+)-α-tocopherol acid succinate (1210 I.U./g), sodium selenite (Alfa Inorganics, Beverly, MA), trolox C (Aldrich Chemical Co., Milwaukee, WI), coenzyme Q₀, coenzyme Q₁₀, (+)-catechin, dimethyl sulfoxide, β-carotene, ascorbic acid palmitate, N-acetylcysteine (Sigma Chemical, St. Louis, MO), canthaxanthin (Roche Vitamin and Fine Chemicals, Nutley, NJ), TBHP (Polysciences, Inc., Warrington, PA), and cupric chloride (CuCl₂) (Merck & Co. Inc., Rahway, NJ), TBHP (Polysciences, Inc., Warrington, PA), and cupric chloride (CuCl₂) (Merck & Co. Inc., Rahway, NJ).

Animals and diets. Male Sprague-Dawley rats (Bantin & Kingman, Fremont, CA), weighing 40–60 g, were adapted for two days before being fed experimental diets. The basal diet (Diet 1) was deficient in vitamin E and selenium with 10% tocopherol stripped corn oil (Teklad Test Diet No. TD 77068, mineral mix No. 170911; Teklad Test Diets, Madison, WI). Animals were housed according to National Institutes of Health guidelines and had free access to deionized water and diets. Diets supplemented with antioxidants were divided into three groups: Diet 2, containing 25 mg vitamin E/kg diet; Diet 3, containing vitamin E, selenium, and the water-soluble antioxidants 50 mg trolox C/kg diet, 200 mg N-acetylcysteine/kg diet, 100 mg coenzyme Q₀/kg diet; and Diet 4, containing vitamin E, selenium, the water-soluble antioxidants and the fat-soluble antioxidants 30 mg coenzyme Q₁₀/kg diet, 45 mg β-carotene/kg diet, 45 mg canthaxanthin/kg diet, 100 mg ascorbic acid palmitate/kg diet. The (+)-catechin/kg diet (100 mg) was added to Diets 3 and 4 as a representative flavonoid. The rats were fed the experimental diets along with distilled water for six weeks.

Preparation of liver homogenates. The rats were decapitated, and livers were immediately dissected and immersed in ice-cold Krebs-Ringer phosphate buffer (pH 7.4). Livers were blotted with filter papers and stored at -22°C. Frozen livers were cut into 0.5 cm³ cubes, and homogenates were prepared by homogenizing 1 g of liver with 9 mL of oxygenated buffer containing glucose (10 mmol, pH 7.4). A motor-driven tissue homogenizer was used.

Oxidation of homogenates and measurement of TBARS. Oxidation of tissue homogenates and measurement of TBARS followed the method described by Tappel and Zalkin (11) with some modifications. Two mL of homogenates were transferred to a 10-mL glass serum bottle containing 3 mL of oxygenated 0.9% NaCl. TBHP or Cu²⁺ for a final concentration of 0.1–1.0 mM, was added to the serum bottle immediately before the reaction began. 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% trichloroacetic acid. The homogenates were centrifuged at 3000 rpm for 2 min at 4°C. After centrifugation, 4 mL of supernatant were reacted with 1.25 mL of 0.75% thiobarbituric acid (freshly prepared) at 100°C for 10 min. The resulting pink color was measured at 530 nm. TBARS were expressed as malondialdehyde equivalents per gram tissue.

The statistical package SAS (SAS Institute Inc., Cary, NC) was used to analyze all data. When significant F-values were obtained using analysis of variance, Duncan’s least square means procedure was used to determine the significant difference (P ≤ 0.05) between treatment means. Results were expressed as means ± standard deviation.

RESULTS

Time course of protection by antioxidants with or without prooxidants. Dietary treatments had no effect on the weight gained by the animals. No exogenous prooxidants were introduced to the incubation vessels during spontaneous lipid peroxidation (time 0). The concentrations of TBARS in liver homogenate from rats fed an antioxidant-deficient diet (Diet 1) rose substantially as incubation time increased (Fig. 1). Addi-

![FIG. 1. Time course of spontaneous lipid peroxidation in liver homogenate showing protection by antioxidants; Diet 1 (basal diet). Liver homogenates were incubated at 37°C. Five timed measurements were taken from homogenates obtained from each rat. The values are expressed as mean for three rats. Curves not sharing a common letter are significantly different from each other at a 95% confidence level. The amounts of thiobarbituric acid-reacting substances (TBARS) are denoted by the alphabetic order with “a” as the largest and “d” as the smallest.](image-url)