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PROTECTION OF HEME PROTEINS BY VITAMIN E, SELENIUM, AND β-CAROTENE AGAINST OXIDATIVE DAMAGE IN RAT HEART, KIDNEY, LUNG AND SPLEEN

H. CHEN[1] and A.L. TAPPEL[1*]

Department of Food Science and Technology, University of California, Davis, CA 95616, U.S.A.

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Effects of the combination of vitamin E, selenium, and β-carotene on oxidative damage to rat heart, kidney, lung, and spleen were studied by measurement of the production of oxidized heme proteins (OHP) during spontaneous and prooxidant-induced oxidation. Male SD rats were fed with a vitamin E and selenium deficient diet or a diet supplemented with vitamin E, selenium, and β-carotene. Homogenates of heart, kidney, lung, and spleen were incubated at 37°C with and without the presence of bromotrichloromethane ($CBrCl_3$). The diet supplemented with antioxidants showed a strong protective effect against oxidative damage to heme proteins during the early stages of both spontaneous and $CBrCl_3$-induced oxidation in contrast to the antioxidant deficient diet. Synergism of multiple antioxygenic nutrients against oxidative damage to various animal tissues is discussed.

KEY WORDS: Heme proteins, Free radicals, oxidative damage, Vitamin E, Selenium, β-carotene

INTRODUCTION

Protection by antioxygenic nutrients against oxidative stress in animal tissues has been definitively established in the last decade[1-9]. Dietary supplementation with multiple antioxygenic nutrients is now being investigated[10]. Studies have indicated that the combination of antioxidants provides stronger protection than individual antioxidants[11-14]. For instance, combining a retinoid and selenium is more effective than either alone in inhibiting chemically induced pancreatic cancer in rats[15].

Oxidative damage of heme proteins involves a redox reaction of the heme group with a one or two electron(s) transfer and the denaturation of the globin structure. The oxidized heme proteins (OHP) can be determined with spectrophotometry[16-19]. A computer aided heme protein spectra analysis program (HPSAP) has been recently employed to quantitatively determine the composition of heme proteins in liver tissues[20]. Quantitation is achieved by matching the calculated spectrum with the experimental spectrum through successive approximations[20-21].

The protective effect of vitamin E, selenium, and β-carotene against oxidative damage to liver tissues has been recently studied by measuring the production of OHP[21]. A diet supplemented with vitamin E, selenium, and β-carotene showed a significant decrease in OHP compared to the supplement of any individual anti-

*To whom the correspondence should be addressed.

oxygenic nutrients. The combination of vitamin E, selenium, and β-carotene is especially effective in preventing oxidative damage in the early stages of oxidation, indicating that synergism may play an important role in both chain-breaking and prevention of oxidation.

In this study we investigated the protective effect of a combination of vitamin E, selenium, and β-carotene against oxidative assaults to heart, kidney, lung and spleen. Rats were fed with a vitamin E and selenium deficient diet and a diet supplemented with vitamin E, selenium, and β-carotene for six weeks. The homogenates of heart, kidney, lung, or spleen were incubated at 37°C with and without the presence of $CBrCl_3$. The absorbance spectra of the homogenate were analyzed with the HPSAP, and the concentrations of OHP were determined. Synergism of multiple antioxygenic nutrients in the protection of tissues against oxidative stress is discussed.

MATERIALS AND METHODS

Chemicals

The chemicals used in this study were tocopherol acid succinate (1210 I.U./gram), trans-β-carotene (95%), dimethyl sulfoxide (Sigma Chemical, St. Louis, MO); sodium selenite (Alfa Inorganics, Beverly, MA); bromotrichloromethane ($CBrCl_3$) (Eastman Kodak Co., Rochester, NY).

Animals and Diets

Male Sprague-Dawley rats (Bantin & Kingman, Fremont, CA) weighing 40–60 g were adapted to their surroundings for 2 days before being fed with experimental diets. The basal diet was vitamin E, selenium, and β-carotene deficient with 10% tocopherol stripped corn oil (Teklad Test Diet #TD 77068, mineral mix #170911, Teklad Test Diets, Madison, WI). Vitamin E as (+)-α-tocopherol (30 I.U./kg), selenium as sodium selenite (0.3 mg/kg), and trans-β-carotene (45 mg/kg) were added to the basal diet. 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. The rats were on the experimental diets and distilled water for six weeks. The concentration of vitamin E in liver was determined and used as an indicator of vitamin E uptake by other tissues such as kidney, heart, lung or spleen. The analysis was conducted at Vitamin and Mineral Laboratory at University of California-Davis using the methods of Bieri *et al.*[22] and Driskell *et al.*[23] The concentrations of vitamin E in livers from rats fed basal diet and diet supplemented with antioxidants were $< 100\,\mu g/dl$ and $169 \pm 15\,\mu g/dl$, respectively. The activity of selenium glutathione peroxidase was determined in a similar diet experiment[10].

Preparation of Tissue Homogenates

The rats were decapitated and heart, kidney, lung, and spleen were immediately dissected and immersed in ice-cold Krebs-Ringer phosphate (KRP) buffer (pH 7.4) to wash away some of the blood. Organs were dried 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.

Oxidative Reactions in Tissue Homogenates

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 cycle/min). $CBrCl_3$ was dissolved in dimethylsulfoxide and added to the serum bottles immediately before the incubation.

Spectrophotometric Measurement of 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 micro-cuvette with a light path of 10 mm and mixed with 0.6 ml glycerol. Four layers of parafilm representing turbidity were used as background to subtract some of the absorbance by turbidity inherent in tissue homogenates. The sample was scanned from 500 nm to 640 nm, and absorbance versus 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 123 (Lotus Development Corp., Cambridge, MA) that contains the micromolar absorbance from 500 to 640 nm of the visible spectra of individual heme proteins. To determine the concentrations of individual heme proteins in a mixture, the experimental visible absorbance versus wavelength at 5 nm intervals from 500 nm to 640 nm was entered into cells in the spreadsheet. To match the experimental spectrum a calculated spectrum is generated. When the best match was achieved, the concentrations of individual heme proteins in the mixture were determined. The detailed information regarding the development of HPSAP, its application, as well as the operation of HPSAP are provided in previous studies[20-21].

Statistical Analysis

The statistical package SAS (SAS Institute Inc., Cary, NC) was used to analyze all data. Results were expressed as means ± standard deviation. Data were analyzed using ANOVA. When significant F values were obtained, the method of least square means was used to determine significant differences ($P \leq 0.05$) between treatment means.

RESULTS

Figure 1 and Table 1 show the application of absorbance spectral measurement and HPSAP to study heme protein oxidation. Oxidative reactions of heme proteins in homogenates of heart, kidney, lung, or spleen caused spectral changes (Figure 1).

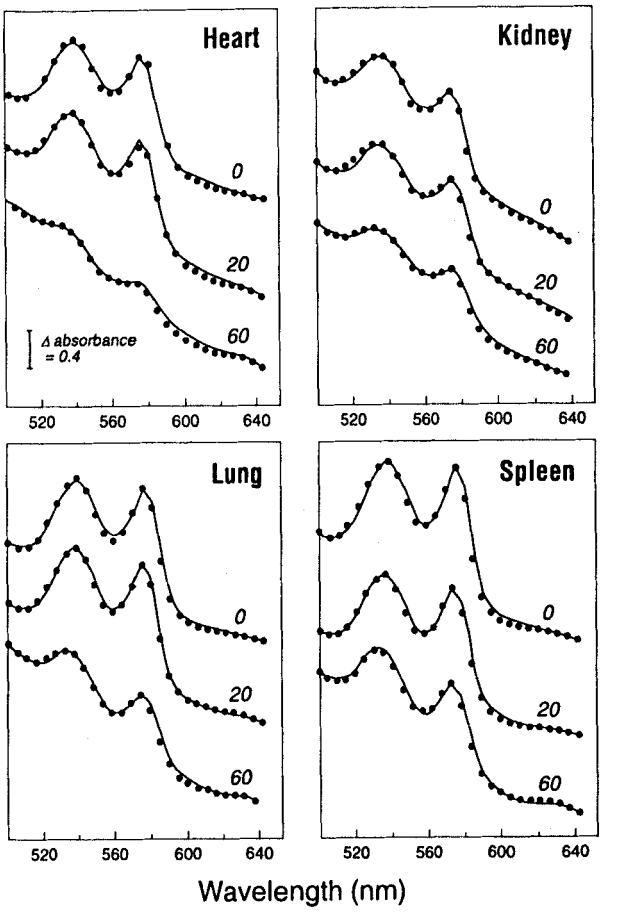

FIGURE 1 Spectral changes of heme proteins in tissue homogenates of heart, kidney, lung, or spleen during spontaneous oxidation (37°C): 0 min; 20 min; and 60 min. ..... experimental spectrum, —— calculated spectrum with HPSAP.

TABLE 1
Production of oxidized heme proteins during spontaneous oxidation measured with HPSAP[1]

Time (min)	Oxidized Heme Protein (Molar%) Kidney	Heart	Lung	Spleen
0	0.0	0.0	0.0	0.0
20	24.6 ± 3.1	24.4 ± 3.7	22.2 ± 1.3	25.6 ± 3.3
60	55.4 ± 10.8	63.1 ± 9.3	64.8 ± 8.2	58.8 ± 5.1

[1]All organs were from rats fed with basal diet for six weeks and the homogenates were incubated at 37°C.

With HPSAP the change in the absorbance spectra can be quantitatively determined (Table 1). The accuracy of the determination is reflected by the fitting between the experimental and calculated spectrum. In general, the calculated spectrum using HPSAP showed good agreement with the experimental spectrum[20-21].

Figure 2 presents the production of OHP in homogenates of kidney, heart, lung, or spleen during incubation with and without the presence of prooxidant and the protection by antioxygenic nutrients against oxidative assaults. In the absence of $CBrCl_3$, incubation of tissues for 20 minutes caused approximately 20–25% oxidative conversion of heme proteins. As the incubation time increased, the concentration of OHP increased rapidly. After 2 h of incubation 65–90% of heme proteins were oxidatively converted. Except for the incubation of 20 min, adding $CBrCl_3$ only showed a little effect on the production of OHP.

The combination of vitamin E, selenium, and β-carotene provides protection against oxidative damage in all organs. In addition, protection is more effective in the early stages of oxidation (20 and 40 min) than in the late stages (1 and 2 h).

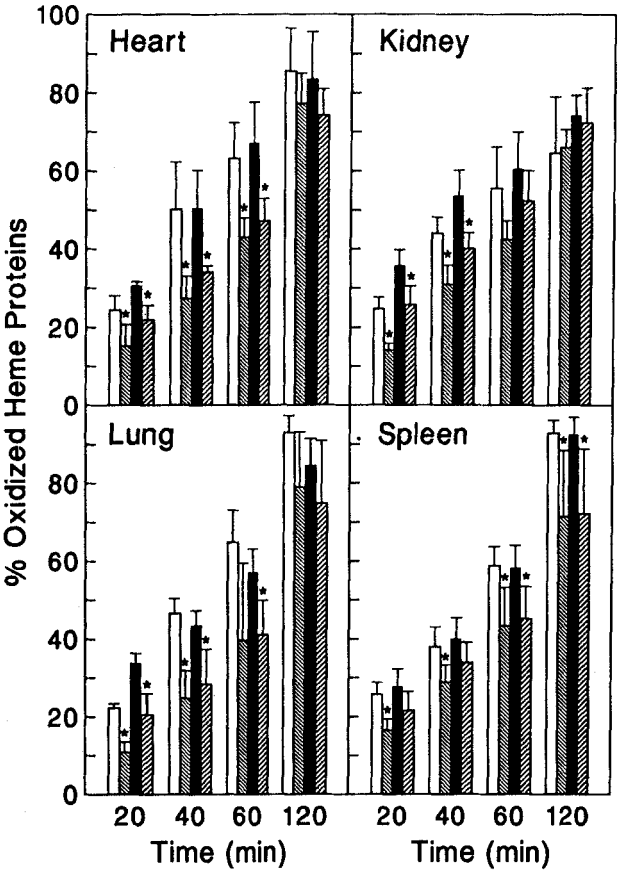

FIGURE 2 Production of oxidized heme proteins during the spontaneous and $CBrCl_3$-induced (0.1 mM) oxidation in heart, kidney, lung, or spleen homogenates. □ basal diet without $CBrCl_3$, ▨ basal diet supplemented with vitamin E, selenium and β-carotene without $CBrCl_3$, ■ basal diet with $CBrCl_3$, ▨ basal diet supplemented with vitamin E, selenium and β-carotene with $CBrCl_3$. Samples were incubated at 37°C and values are expressed as mean ± SD for 4 rats. The mean values marked with * were significantly smaller than the mean of respective basal diet group at a 95% confident level.

DISCUSSION

Heme proteins are highly susceptible to oxidative stress and are readily converted to their oxidized forms[16-19]. OHP can be a useful index of oxidative damage in animal tissues[20]. However, under conditions close to physiological, such as tissue homogenates, the quantitative measurement of oxidized heme proteins is quite difficult due to the turbidity caused by biological tissues. In addition, spectra obtained from tissue homogenate are usually complex due to the presence of both reduced and oxidized heme proteins. The HPSAP analysis is a part of the solution for these problems. As shown in Figure 1 and Table 1, the concentration of oxidized heme proteins in a mixture can be determined using HPSAP. The merits and strengths of HPSAP in the quantitation of heme proteins in biological samples have been discussed[20-21].

The combination of vitamin E, selenium, and β-carotene showed a protective effect on heart, kidney, lung, and spleen against oxidation of heme proteins. The most significant feature of the synergism of vitamin E, selenium, and β-carotene is its effectiveness in the inhibition of early stages of oxidation. In lung tissue, for instance, antioxygenic nutrients reduced production of oxidized heme proteins by more than 50% during the early periods of spontaneous oxidation (20 and 40 min).

Except for the short period of incubation (20 min), adding $CBrCl_3$ did not promote increase of OHP compared to the that produced during spontaneous oxidation. There are two possible explanations for this observation. First, the concentration of $CBrCl_3$ is relatively low (0.1 mM) so the damage caused by $CBrCl_3$ is very small. Secondly, the deleterious effect of $CBrCl_3$ comes from its activation by microsomal oxidase systems. For instance, in the liver tissues, $CBrCl_3$ is activated by cytochrome P-450 systems. In kidney, heart, lung and spleen tissues, such enzyme systems are not as active as those in liver, so the effect of $CBrCl_3$ on heme protein oxidation is limited.

Vitamin E is particularly effective in trapping free radicals thus inhibiting chain reactions and selenium-glutathione peroxidase is essential in the conversion of hydroperoxides to stable non-radical products[3-4,7-8,24]. Beta-carotene effectively scavenges singlet oxygen and other reactive oxygenic species[8-9]. Vitamin E and β-carotene are primary antioxidants in cellular membranes while GSH peroxidase is a cytosolic antioxidant in the aqueous phase[11]. Since oxidative damage of proteins is usually accompanied with other oxidative processes such as lipid peroxidation[25-26], various reactive oxygenic species generated from these processes could have a great impact on heme protein oxidation. Even though the oxidation of heme proteins occurs mainly in the aqueous phase, reactive oxygenic species generated from membranes are also likely to attack heme proteins. The combination of vitamin E, selenium and β-carotene would be capable of interacting with various types of free radicals in both cellular membranes and aqueous phase of tissue. As a result, the synergism of both aqueous and membranous antioxidants may enhance the protection against oxidative damage to heme proteins over that of individual antioxidants.

In this study the synergistic interaction among vitamin E, selenium, and β-carotene showed a significant inhibitory effect against the spontaneous and prooxidant-induced oxidation of heme proteins in heart, kidney, lung, and spleen tissues. The protection is more effective during the early stage of the oxidative reaction.

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References

1. Burton, G.W. and Ingold, K.U. (1989) Vitamin E as an *in Vitro* and *in Vivo* antioxidant. In *Vitamin E: biochemistry and health implications* (eds. A.T. Diplock, L.J. Machlin, L. Packer and W.A. Pryor). The New York Academy of Sciences, New York, pp. 7–22.
2. G.W. Burton, A. Joyce and K.U. Ingold (1982) First proof that vitamin E is a major lipid soluble, chain-breaking antioxidant in human blood plasma. *Lancet*, **2**, 327.
3. G.W. Burton, K.H. Cheeaman, T. Doba and K.U. Ingold (1983) Vitamin E as an antioxidant *in Vitro* and *in Vivo*. In *Ciba Foundation Symposium 101. Biology of vitamin E.* Pitman Press, London, pp. 4–18.
4. A.L. Tappel (1980) Vitamin E and selenium protection from *in vivo* lipid peroxidation. *New York Academy Science*, **355**, 18–31.
5. J.W. Forstrom, J.J. Zakowski and A.L. Tappel (1978) Identification of the catalytic site of rat liver glutathione peroxidase as selenocysteine. *Biochemistry*, **17**(13), 2639–2644.
6. N.I. Krinsky (1989) Antioxidant functions of carotenoids. *Free Radical Biology and Medicine*, **7**, 617–635.
7. E. Niki (1991) Antioxidant compounds. In *Oxidative damage and repair: chemical, biological and medical aspects* (ed. K.J.A. Davies), Pergamon Press, New York, pp. 57–64.
8. G.W. Burton and K.U. Ingold (1989) Mechanisms of antioxidant action: preventive and chain-breaking antioxidants. In *CRC Handbook of Free Radicals and Antioxidants in Biomedicine (II)* (eds. J. Miquel, A.T. Quintanilha and H. Weber), CRC Press, Boca Raton, FL, pp. 29–44.
9. G.W. Burton and K.U. Ingold (1984) β-carotene: an unusual type of lipid antioxidant. *Science*, **224**, 581–590.
10. B. Leibovitz, M-L. Hu and A.L. Tappel (1990) Dietary supplements of vitamin E, β-carotene, coenzyme Q_{10} and selenium protect tissues against lipid peroxidation in rat tissue slices. *Journal of Nutrition*, **120**, 97–104.
11. A.L. Tappel (1992) Combinations of vitamin E and other antioxygenic nutrients in protection of tissues. In *Vitamin E in health and diseases* (eds. L. Packer and J. Fuchs), Marcel Dekker Inc., New York, pp. 313–325.
12. R. Zamora, F.J. Hidalgo and A.L. Tappel (1990) Comparative antioxidant effectiveness of dietary β-carotene, vitamin E, selenium and coenzyme Q_{10} in rat erythrocytes and plasma. *Journal of Nutrition*, **121**, 50–56.
13. C. Ip (1988) Feasibility of using lower doses of chemopreventive agents in a combination regimen for cancer protection. *Cancer Letters*, **39**, 239–246.
14. C.E. Cross, B. Halliwell, E.T. Borish, W.A. Pryor, B.N. Ames, R.L. Saul, J.M. McCord and D. Harman (1987) Oxygen radicals and human disease. *Ann International Medicine*, **107**, 526–545.
15. T.J. Curphey, E.T. Kuhlmann, B.D. Roebuck and D.S. Longnecker (1988) Inhibition of pancreatic and liver carcinogenesis in rats by retinoid- and selenium-supplemented diets. *Pancreas*, **3**, 36–40.
16. D. Chiu, B. Lubin and S.B. Shohet (1982) Peroxidative reactions in red blood cell biology. In *Free radicals in biology. Vol. V.* (ed. W.A. Pryor), Academic Press, New York, pp. 115–160.
17. G.M. Wagner, B.H. Lubin and D. T-Y. Chiu (1988) Oxidative damage to red blood cells. In *Cellular antioxidant defense mechanisms. Vol. I* (ed. C.K. Chow), CRC Press, FL, pp. 185–195.
18. C.C. Winterbourn (1985) Free-radical production and oxidative reactions of hemoglobin. *Environmental Health Perspective*, **64**, 321–330.
19. C.C. Winterbourn (1990) Oxidative reactions of hemoglobin. In *Methods in Enzymology. Vol. 186* (eds. L. Packer and A.N. Glazer). Academic Press, New York, pp. 265–272.
20. H. Chen, H., A.L. Tappel and C.R. Boyle (1993) Oxidation of heme proteins as a measure of oxidative damage to liver tissue slices. *Free Radical Biology and Medicine*, **14**, 509–517.
21. H. Chen, L.J. Pellett, H.J. Andersen and A.L. Tappel (1993) Protection by vitamin E, selenium, and β-carotene against oxidative damage in rat liver slices and homogenate. *Free Radical Biology and Medicine*, **14**, 473–482.

22. J.G. Bieri, T.J. Tolliver and G.L. Catignani (1979) Simultaneous determination of α-tocopherol and retinol in plasma or red cells by high pressure liquid chromatography. *Am. J. Clin. Nutr.*, **32**, 2143–49.
23. W.J. Driskell, J.W. Nees, C.C. Bryant, and M.M. Bashor (1982) Measurement of vitamin A and vitamin E in human serum by high performance liquid chromatography: *J. Chromatography*, **231**, 439–44.
24. H. Sies (1985) Oxidative stress: introductory remarks. In *Oxidative stress* (ed. H. Sies). Academic Press, New York, pp. 1–10.
25. R.E. Pacifici and K.J.A. Davies (1990) Protein degradation as an index of oxidative stress. In *Methods in Enzymology. Vol. 186.* (eds. L. Packer and A.N. Glazer), Academic Press, New York, pp. 485–502.
26. K.J.A. Davies (1988) Oxidative stress causes protein degradation and lipid peroxidation by different mechanisms in red blood cells. In *Lipid peroxidation in biological systems* (ed. A. Savanian), American Oil Chemists' Society, Champaign, IL, pp. 100–116.

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