Excess Dietary Vitamin E Lowers the Activities of Antioxidative Enzymes in Erythrocytes of Rats Fed Salmon Oil

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ABSTRACT In vitro studies suggest that high vitamin E supplementation has prooxidative activity, but very few studies have investigated this effect in vivo. We investigated the effect of excess vitamin E on the antioxidative status of rat erythrocytes and indicators of hemolysis. Six groups of growing male Sprague-Dawley rats were fed purified diets with three different vitamin E doses [100, 1000 and 10,000 mg all-rac-α-tocopheryl acetate (TA)/kg diet] and two different dietary fats (salmon oil and lard) for 8 wk. The rats whose diet contained salmon oil and 10,000 mg TA/kg had lower activities of superoxide dismutase (P < 0.05), glutathione peroxidase (P < 0.05), catalase (P < 0.05) and a lower concentration of glutathione (P < 0.05) in the erythrocyte cytosol than rats whose diet contained 100 mg TA/kg. The concentration of free hemoglobin and the binding capacity of haptoglobin in plasma, both indicators of in vivo hemolysis, did not differ between rats fed the salmon oil diet with 100 or 10,000 mg TA/kg. In the rats whose diet contained lard, the activities of antioxidant enzymes in erythrocytes and indicators of in vivo hemolysis were independent of the dietary vitamin E concentration. The results of the study suggest that an excessive vitamin E intake, when combined with salmon oil in the diet, lowers the activities of antioxidant enzymes in erythrocytes without affecting in vivo hemolysis. J. Nutr. 132: 3400–3404, 2002.

KEY WORDS: • rats • erythrocytes • vitamin E • hemolysis • antioxidative enzymes

Vitamin E, a lipid-soluble antioxidant, can react with organic peroxides due to the presence of the phenolic hydroxy group on the chroman ring of the molecule, thus interrupting the chain reaction of lipid peroxidation (1,2). This reaction involves the formation of tocopheroxyl radicals, which are regenerated by means of hydrogen donors (1,2). If this reduction is incomplete, the tocopheroxyl radicals can initiate oxidative processes (3). In vitro studies with micellar suspensions (3) and isolated LDL (4), high doses of vitamin E had prooxidative effects. The cause of this prooxidative activity lies in the reaction of the tocopheroxyl radical with other peroxyl radicals or with polyunsaturated fatty acids (PUFA)3 in the LDL. This reaction leads to an accumulation of hydroperoxides with a conjugated diene structure (3). There are at present very few studies on potential prooxidative effects of high vitamin E supplements in vivo. The present study was therefore undertaken to investigate potential prooxidative effects of high vitamin E doses in a rat model in vivo.

Erythrocytes are highly sensitive to oxidative stress because of their high concentrations of hemoglobin and oxygen. It has been shown that under conditions of oxidative stress, activities of antioxidant enzymes such as superoxide dismutase (SOD), glutathione peroxidase (GSH-Px) and catalase increase (5–8). In the present study, we examined the activities of various antioxidant enzymes of erythrocytes as an indicator of oxidative stress. In severe oxidative stress, as caused for instance by a deficiency of vitamin E, the susceptibility of erythrocytes to hemolysis is enhanced (9). To test whether high dietary vitamin E supplementation could affect erythrocyte hemolysis, we also examined some indicators of in vivo hemolysis such as the concentration of free hemoglobin and the binding capacity of haptoglobin in plasma.

It is likely that potential prooxidative effects of excessive vitamin E doses also depend on the type of dietary fat. Feeding fats with a high content of PUFA such as fish oil increases concentrations of PUFA in the erythrocyte membrane and makes the membrane more prone to oxidation (10). We therefore used two different fats in this study, lard with a relatively low concentration, and fish oil with a high concentration of highly unsaturated fatty acids.

MATERIALS AND METHODS

Animals. Adult male Sprague-Dawley rats (n = 60) with an initial body weight of 62.4 (±4.2 g, SD), obtained from Charles River GmbH (Sulzfeld, Germany), were randomly assigned to one of six groups of 10 rats each. They were housed individually in Macrolon...
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Dietary analysis. The basal diets consisted of (g/kg diet): cornstarch (398), cellulose (49.9), fat (100), vitamins and minerals (50) and dl-methionine (2). Ketones and vitamins with the exception of vitamin E were supplemented in accordance with recommendations of the AIN (11) for rat diets. As dietary fat, either salmon oil (obtained from Caelo, Hilden, Germany) or lard (obtained from Laru, Bottrop, Germany) was used. The basal salmon oil diets contained 9.4 mg α-tocopherol/kg and the basal lard diets contained 1.3 mg α-tocopherol/kg. The diets were supplemented individually with all-rac-α-tocopheryl acetate (TA; Merck, Darmstadt, Germany) to reach final vitamin E levels corresponding to 100, 1000 and 10,000 mg TA/kg diet. We assumed that the activity of 1 mg α-tocopherol originally present in the diet is equal to that of 1.49 mg TA (12). TA was supplemented at the expense of cellulose. The concentration of cellulose varied from 49.9 g/kg diet in the diets with 100 mg TA/kg diet to 40 g/kg diet in the diets with 10,000 mg TA/kg diet.

The fatty acid composition of the dietary fats is shown in Table 1. The peroxide values of the dietary fats, which were extracted from the diets with a mixture of hexane and isopropanol (3:2 according to (13)), were independent of the dietary vitamin E concentration. Peroxide values of the dietary fats used, measured by official methods (14), were 9.5, 9.3 and 9.6 mM LO2/kg fat in the salmon oil diets containing 100, 1000 and 10,000 mg TA/kg and 2.4, 2.5 and 2.5 mM LO2/kg fat in the lard diets containing 100, 1000 and 10,000 mg TA/kg, respectively.

The diets were prepared by mixing the dry components with fat and water and subsequent freeze drying. The residual water in the diets was <5 g/100 g diet. Diets were administered in restricted amounts to standardize the feed intake. Feeding took place once daily during the experiment from 6.4 g to 17.6 g. Water was freely available from nipple drinkers. The diets were fed for 8 wk.

Sample collection. After completion of the feeding periods, the rats were starved for 12 h and killed by decapitation under light anesthesia with diethyl ether. Blood was collected into heparinized polyethylene tubes. Plasma and erythrocytes were obtained by centrifugation of the blood (1100 × g, 10 min). Erythrocytes were washed three times with 9 g/L sodium chloride solution. Plasma and erythrocytes were stored at −20°C until analysis.

Antioxidant enzymes in erythrocytes. Antioxidant enzymes were measured in hemolysates. The activity of glutathione peroxidase was determined with t-butyl hydroperoxide at 25°C (15). One unit of GSH-Px activity is defined as 1 μmol reduced β-nicotinamide adenine dinucleotide phosphate oxidized/min. Superoxide dismutase activity was determined with pyrogallol as the substrate (16). One unit of SOD activity is defined as the amount of enzyme required to inhibit the autoxidation of pyrogallol by 50%. Catalase activity was determined at 25°C using hydrogen peroxide as the substrate according to the method of Aebi (17). One unit of catalase activity is defined as the amount consuming 1 μmol hydrogen peroxide/min. Glucose-6-phosphate dehydrogenase (G6PDH) activity was determined by a method of Deutsch (18). One unit of G6PDH activity is defined as the amount of enzyme required to reduce 1 μmol β-nicotinamide adenine dinucleotide phosphate/min. The concentration of glutathione in hemolysates was measured with glutathione reductase and Ellman’s reagent (19). Calibration was performed using a standard curve.

Indicators of in vivo hemolysis of erythrocytes. Plasma concentration of free hemoglobin (Hb) was measured with a kit reagent (catalog no. 527) obtained from Sigma (Taufkirchen, Germany). The activities of lactate dehydrogenase (LDH) and acid phosphatase were determined with kits (Ecoline 15; Merkotest 330) obtained from Merck. The binding capacity of haptoglobin (Hp) was determined with a Hewlett-Packard HPLC system (Walldiron, Germany) consisting of a HP 1100 pump and a HP-1100 ultraviolet detector (20). Hp was added to the plasma in a defined concentration. The Hp-Hb complex was separated on a gel filtration column (type GF 250, 250 mm length, 4.6 mm i.d.) with 0.2 mol/L sodium hydroxide (pH 7.2) as eluent at a flow rate of 0.9 mL/min and detected at a wavelength of 418 nm. The binding capacity of Hp in plasma was expressed as g bound Hp/l plasma.

Fatty acids and cholesterol in erythrocyte membranes. Total lipids of erythrocytes were extracted with a mixture of n-hexane and isopropanol (3:2, v/v) (13). The fatty acid composition of erythrocyte membrane was determined by gas chromatography. Total lipids were transmethylated into fatty acid methyl esters (FAME) with trimethylsilyl trifluoroacetamide (Merck). FAME were separated by using a gas chromatographic system (HP 5890, Hewlett-Packard) fitted with an automatic on-column injector, a flame ionization detector and a polar capillary column (FFAP, 30 m, 0.53 mm i.d., Macherey and Nagel, Düren, Germany). Helium was used as the carrier gas at a constant flow rate of 4.7 mL/min. The following oven temperature program was used: 100°C held for 1 min, increased to 160°C at 40°C/min, held for 5 min at 160°C, increased to 220°C at 2°C/min, held for 10 min. FAME were detected by flame ionization and identified by comparing their retention times with those of individually purified standards (21). For measurement of erythrocyte membrane cholesterol, lipids of the extract were dissolved in Triton X-100 (22). Cholesterol was determined using a commercially available enzymatic reagent kit (Ecoline 25, Merck).

Tocopherol concentrations. Concentrations of individual tocopherols in plasma, erythrocytes and dietary fats were determined in a Hewlett-Packard HPLC system consisting of a HP 1100 pump and a HP-1100 fluorescence detector (23). Samples were mixed with 1 mL of 10 g/L pyrogallol solution (in ethanol, absolute) and 150 μL of saturated sodium hydroxide solution. This mixture was heated for 30 min at 70°C, and tocopherols were extracted with n-hexane. Individual tocopherols of the extracts were separated isocratically using a mixture of n-hexane and 1,4 dioxane (94:6, v/v) as mobile phase at a flow rate of 1 mL/min and a LiChrosorb Si-60 column (5-μm particle size, 250 mm length, 4 mm i.d., Merck) and detected by fluorescence (excitation wavelength: 295 nm, emission wavelength: 330 nm).

Statistics. Results were analyzed by two-way ANOVA. Classification factors were vitamin E concentration and fat type, as well as their interaction. For significant F-values, individual means were compared by Fisher’s multiple range test. Means were considered significantly different at P < 0.05. Values in the text are means ± SD.

RESULTS

Food intake and body weight gain. Neither the dietary vitamin E concentration nor the dietary fat affected food intake or weight gain (5.9 ± 0.2 g/d; n = 60) of the rats. The food intake of each rat was 17.9 g/d due to the standardized intake protocol used.

α-Tocopherol in plasma and erythrocytes. The tocopherol concentrations in both plasma and erythrocytes increased only moderately with rising vitamin E concentrations (Fig. 1).

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Fatty acid composition of the dietary fats1</th>
<th>Salmon oil</th>
<th>Lard</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fatty acid</td>
<td>g/100 g fatty acids</td>
<td>g/100 g fatty acids</td>
<td></td>
</tr>
<tr>
<td>Total saturated fatty acids</td>
<td>27.1</td>
<td>47.6</td>
<td></td>
</tr>
<tr>
<td>Total monounsaturated fatty acids</td>
<td>39.5</td>
<td>39.2</td>
<td></td>
</tr>
<tr>
<td>Total polyunsaturated fatty acids</td>
<td>29.1</td>
<td>11.9</td>
<td></td>
</tr>
<tr>
<td>(n-6) polyunsaturated fatty acids</td>
<td>3.1</td>
<td>10.2</td>
<td></td>
</tr>
<tr>
<td>(n-3) polyunsaturated fatty acids</td>
<td>23.8</td>
<td>1.3</td>
<td></td>
</tr>
<tr>
<td>Unsaturation index2</td>
<td>1.73</td>
<td>0.64</td>
<td></td>
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</tbody>
</table>

1 Fatty acids in quantities <0.05 g/100 g were not considered.
2 Average number of double bonds/mol of fatty acids.
Antioxidative enzymes in erythrocytes. There were significant interactions between dietary fat and dietary vitamin E for the activities of SOD, GSH-Px, catalase and G6PDH. In rats whose diet contained salmon oil, the activities of all of these enzymes declined with increasing dietary vitamin E concentrations (Table 2). In the rats whose diet contained 10,000 mg TA/kg, the activities of all of these enzymes were significantly lower than in rats whose diet contained 100 mg TA/kg. In rats whose diet contained lard, the activities of SOD, GSH-Px, catalase and G6PDH were independent of the dietary vitamin E concentration.

Glutathione in erythrocytes. There was a significant interaction between dietary fat and vitamin E for the concentration of glutathione in erythrocytes (Table 2). Within the salmon oil groups, rats fed the diet containing 10,000 mg TA/kg had a significantly lower concentration than those fed diets containing 100 or 1000 mg TA/kg. Within the lard-fed groups, the concentration of glutathione in erythrocytes was significantly higher in rats fed the diet containing 1000 mg TA/kg than in rats fed the diet containing 100 mg TA/kg diet with an intermediate activity in those fed the lard diet containing 10,000 mg TA/kg.

In vivo hemolysis. Within the salmon oil groups, the concentration of free Hb in plasma was lower in rats fed diets containing 1000 mg TA/kg than in those fed diets containing 100 or 10,000 mg TA/kg (Table 3). Within the lard groups, the concentration of free Hb was not affected by dietary vitamin E.

The binding capacity of Hp was lower in rats whose diet contained salmon oil than in rats whose diet contained lard. Within the salmon oil groups, the binding capacity of Hp was independent of the dietary vitamin E concentration, whereas in the lard-fed rats, it was higher in those fed the diet containing 10,000 mg TA/kg than in those fed diets containing 100 or 1000 mg TA/kg.

For the activities of acid phosphatase and LDH in plasma, there were significant interactions between dietary fat and vitamin E. Within the salmon oil groups, the activities were significantly higher in rats fed diets containing 1000 or 10,000 mg TA/kg than in those fed the diet containing 100 mg TA/kg. Within the lard groups, the activity of acid phosphatase was significantly lower in rats fed diets containing 1000 or 10,000 mg TA/kg than in those fed diets containing 100 mg TA/kg.

In the rats whose diet contained 10,000 mg TA/kg diet, the concentration of α-tocopherol in plasma was approximately twice as high as in the rats whose diet contained 100 mg TA/kg diet. The concentration of α-tocopherol in the erythrocytes was ~50% higher in the rats whose diet contained 10,000 mg TA/kg than in those whose diet contained 100 mg TA/kg. Moreover, concentrations of α-tocopherol in plasma and erythrocytes were generally higher in rats whose diet contained lard than in those whose diet contained salmon oil.
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TABLE 3

Concentration of free hemoglobin, activities of acid phosphatase and of lactate dehydrogenase and binding capacity of haptoglobin in plasma of rats fed diets containing 100, 1000 or 10,000 mg
all-rac-α-tocopheryl acetate/kg diet with salmon oil or lard

<table>
<thead>
<tr>
<th>Fat type</th>
<th>Vitamin E, mg all-rac-α-tocopheryl acetate/kg diet</th>
<th>Salmon oil</th>
<th>Lard</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>100</td>
<td>1000</td>
<td>10,000</td>
</tr>
<tr>
<td>Free hemoglobin, g/L</td>
<td>254 ± 147a</td>
<td>136 ± 60b</td>
<td>259 ± 146a</td>
</tr>
<tr>
<td>Acid phosphatase, U/g protein</td>
<td>0.47 ± 0.17b</td>
<td>0.66 ± 0.20a</td>
<td>0.76 ± 0.16a</td>
</tr>
<tr>
<td>Lactate dehydrogenase, U/g protein</td>
<td>4.20 ± 1.09b</td>
<td>6.89 ± 2.48a</td>
<td>6.17 ± 1.39a</td>
</tr>
<tr>
<td>Binding capacity of haptoglobin, g Hb/L</td>
<td>7.8 ± 1.2c</td>
<td>6.9 ± 1.3c</td>
<td>7.7 ± 0.9c</td>
</tr>
</tbody>
</table>

1 Values are means ± sd, n = 10. Means in a row without a common letter differ significantly, P < 0.05.

This would explain the lowered activities of antioxidative enzymes in the rats whose diet contained excessive concentrations of vitamin E. In another study with rats fed a fish oil diet, increasing the vitamin E supply reduced activities of antioxidative enzymes in the liver and in muscle (5). Further research is required, however, to elucidate the molecular interactions among oxidative stress, vitamin E and the expression of antioxidative enzymes in erythrocytes.

To determine whether reduced activities of antioxidative enzymes in erythrocytes caused by excess dietary vitamin E could affect hemolysis of erythrocytes, we measured various indicators of hemolysis, i.e., the activities of LDH and acid phosphatase in the blood, the concentrations of free Hb and the binding capacity of Hp. The finding that the concentrations of free Hb and the binding capacity of Hp were not raised in rats fed the fish oil diet containing excess vitamin E suggests that their rate of hemolysis was not elevated. A marked increase in hemolysis would be expected to result in a higher concentration of free Hb and a lower binding capacity of Hp (31). The observation that the activities of LDH and acid phosphatase were increased does not by itself imply an increased hemolysis rate because these are relatively unspecific parameters. These enzymes occur not only in erythrocytes but also in other tissues. The results do not suggest that megadoses of vitamin E cause increased hemolysis in vivo.

High dose vitamin E therapy in humans can reach daily doses in excess of 500 mg, equivalent to dietary vitamin E concentrations of up to 1000 mg/kg diet. In our study, doses of 1000 mg TA/kg diet, although without effect on erythrocyte hemolysis, did significantly lower the activities of GSH-Px, catalase and G6PDH in erythrocytes. The results of this study with rats cannot be extrapolated directly to humans. Nevertheless, the study suggests that high dose vitamin E therapy could affect enzymes of the antioxidative system of erythrocytes.

DISCUSSION

This study was undertaken to investigate the effect of the dietary vitamin E supply on the antioxidative status of rat erythrocytes. Even the lowest dose of vitamin E used in this experiment was well in excess of the requirement for rats. Assuming a specific vitamin E requirement for unsaturated fatty acids as suggested by Muggli (24), we estimated the vitamin E requirement of rats fed the salmon oil diets to be 52 mg TA/kg diet, and that of rats fed the lard diets to be 15 mg TA/kg diet. The highest dose (10,000 mg TA/kg diet), which is equivalent to a mean vitamin E intake of 0.5 mg TA/g body, was below the toxic level, which is >2 mg TA/g body (25,26).

In agreement with other studies (27), an excessive vitamin E supply therefore had no adverse effects on feed intake or growth, regardless of the type of fat consumed.

Megadoses of vitamin E lowered the activities of antioxidative enzymes in the erythrocytes and lowered the concentration of glutathione in rats fed a diet with a high proportion of PUFAs. Because this effect of excess vitamin E occurred only in rats fed salmon oil and not in those fed lard, there may be a link between the susceptibility of the erythrocyte membranes to oxidation and the type of dietary fat. This and other studies (10,28) have shown that feeding fish oil leads to an accumulation of highly unsaturated, oxidation-susceptible (n-3) PUFAs in erythrocyte membrane lipids. Furthermore, oxidative stress in various tissues leads to an enhanced expression of antioxidative enzymes (29,30). We suggest that the formation of lipid peroxides in membranes of immature erythrocytes in the bone marrow of the rats whose diet contained fish oil was reduced by increased concentrations of vitamin E. As a consequence, the expression of antioxidative enzymes in immature erythrocytes could also have been reduced in these rats.

LITERATURE CITED


