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 peroxidase (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.

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 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
cages in a room controlled for temperature (22 ± 2°C), humidity and light (12-h light:dark cycle). All animal procedures described followed established guidelines for the care and handling of laboratory animals and were approved by the regional council of Saxony-Anhalt.

Diets. According to a bifactorial experimental design, six diets were used, differing in their type of fat and their vitamin E concentration. The basal diets consisted of (g/kg diet): cornstarch (398), casein (200), saccharose (200), fat (100), cellulose (50), vitamins and minerals (50) and β-methionine (2). Minerals 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, Hiliden, 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 or 10,000 mg TA/kg diet. We assumed that the activity of 1 mgα-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 lipids of erythrocytes were measured in hemolysates. The activity of glutathione peroxidase was determined with 1 mM α-phenyl-Nethyl-p-nitrophenyl-phosphate at 25°C using a gas chromatographic system (HP 5890, Hewlett-Packard) fitted with an automatic on-column injector, a flame ionization detector and a polycapillary 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, then 160°C held for 10 min. FAME were detected by a method of Deutsch (18). One unit of G6PDH 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). Catalase activity was measured in a standard curve.

TABLE 1

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

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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 lipids was determined by gas chromatography. Total lipids were transmethylated into fatty acid methyl esters (FAME) with trimethylsulfonium hydroxide. FAME were separated on a fused silica capillary column using a gas chromatographic system (HP 5890, Hewlett-Packard) fitted with an automatic on-column injector, a flame ionization detector and a polycapillary 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, then 160°C held for 10 min. FAME were detected by a method of Deutsch (18). One unit of catalase 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). Catalase activity was measured in a standard curve.

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).
In the rats whose diet contained 10,000 mg TA/kg diet, the concentration of A-tocopherol in plasma was approximately twice as high as in the rats whose diet contained 100 mg TA/kg diet. The concentration of A-tocopherol in plasma and 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 A-tocopherol in plasma and erythrocytes were generally higher in rats whose diet contained lard than in those whose diet contained salmon oil.

**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 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 1000 mg TA/kg.

*Table 2*

*Activities of superoxide dismutase, glutathione peroxidase, catalase and glucose-6-phosphate dehydrogenase and concentration of glutathione in erythrocytes 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>
<th>Salmon oil</th>
<th>Lard</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>100</td>
<td>1000</td>
<td>10,000</td>
<td>100</td>
<td>1000</td>
</tr>
<tr>
<td><strong>μg/mg protein</strong></td>
<td></td>
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<tr>
<td>Superoxide dismutase</td>
<td>5.29 ± 1.47&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.52 ± 1.62&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.59 ± 0.76&lt;sup&gt;b&lt;/sup&gt;</td>
<td>5.67 ± 1.27&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.78 ± 1.29&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Glutathione peroxidase</td>
<td>1.32 ± 0.52&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.77 ± 0.23&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>0.55 ± 0.08&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.82 ± 0.24&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>0.94 ± 0.53&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td>Catalase</td>
<td>273 ± 16&lt;sup&gt;a&lt;/sup&gt;</td>
<td>133 ± 69&lt;sup&gt;b&lt;/sup&gt;</td>
<td>83 ± 16&lt;sup&gt;b&lt;/sup&gt;</td>
<td>88 ± 29&lt;sup&gt;b&lt;/sup&gt;</td>
<td>153 ± 49&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Glucose-6-phosphate dehydrogenase</td>
<td>17.7 ± 6.7&lt;sup&gt;a&lt;/sup&gt;</td>
<td>12.0 ± 4.7&lt;sup&gt;b&lt;/sup&gt;</td>
<td>10.8 ± 4.0&lt;sup&gt;b&lt;/sup&gt;</td>
<td>10.0 ± 4.8&lt;sup&gt;b&lt;/sup&gt;</td>
<td>9.7 ± 3.6&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>μmol/g protein</strong></td>
<td></td>
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<tr>
<td>Glutathione</td>
<td>9.3 ± 2.6&lt;sup&gt;b&lt;/sup&gt;</td>
<td>9.9 ± 2.8&lt;sup&gt;b&lt;/sup&gt;</td>
<td>6.2 ± 1.4&lt;sup&gt;c&lt;/sup&gt;</td>
<td>10.8 ± 2.7&lt;sup&gt;b&lt;/sup&gt;</td>
<td>14.4 ± 3.0&lt;sup&gt;a&lt;/sup&gt;</td>
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</tbody>
</table>

<sup>1</sup> Values are means ± so, n = 10. Means in a row without a common letter differ significantly, *P* < 0.05.
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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 lard1

<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>
<th>Salmon oil</th>
<th>Lard</th>
</tr>
</thead>
<tbody>
<tr>
<td>Free hemoglobin, g/L</td>
<td>254 ± 147a</td>
<td>136 ± 60b</td>
<td>259 ± 146a</td>
<td>179 ± 81ab</td>
<td>127 ± 26b</td>
</tr>
<tr>
<td>Acid phosphatase, μg protein</td>
<td>0.47 ± 0.17b</td>
<td>0.66 ± 0.20a</td>
<td>0.76 ± 0.16a</td>
<td>0.63 ± 0.19a</td>
<td>0.49 ± 0.14b</td>
</tr>
<tr>
<td>Lactate dehydrogenase, μg protein</td>
<td>4.20 ± 1.99b</td>
<td>6.89 ± 2.48a</td>
<td>6.71 ± 1.39a</td>
<td>6.19 ± 1.00a</td>
<td>5.61 ± 2.10ab</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>
<td>9.9 ± 1.9b</td>
<td>9.7 ± 1.8b</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 antioxidant 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, 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 for unsaturated fatty acids as suggested by Muggli (24), we estimated the amount of vitamin E used in this experiment to be 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 for unsaturated fatty acids as suggested by Muggli (24), we estimated the amount of vitamin E used in this experiment to be 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 for unsaturated fatty acids as suggested by Muggli (24), we estimated the amount of vitamin E used in this experiment to be well in excess of the requirement for rats.

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 PUFA. 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 lipids 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


