The present study was designed to assess plasma and erythrocyte vitamin E concentrations in 57 asymptomatic hypercholesterolemic (HC) men compared with 56 normocholesterolemic (NC) men. Vitamin E concentrations were determined by using a reversed-phase HPLC method. Compared with NC subjects, HC men had a significantly lower red blood cell (RBC) vitamin E content in spite of their normal plasma vitamin E concentration. This study demonstrates that total plasma vitamin E concentration is not a suitable predictor of cell vitamin E status and suggests an abnormal transfer of tocopherol between plasma and RBCs in HC men. Moreover, the RBCs of HC men were more susceptible to a peroxidative stress. The strong correlation between RBC susceptibility to oxidation and RBC vitamin E content suggests that the low RBC vitamin E content found in HC men has physiological consequences on the RBC oxidation.

INDEXING TERMS: \(\alpha\)-tocopherol • red blood cells • atherosclerosis • HPLC • antioxidants • peroxidative stress

An increasing interest in the role of oxidative stress as a potential initiating factor in atherosclerosis has been observed in recent years [1, 2]. A variety of antioxidant defense systems in the human body are able to detoxify prooxidants and scavenge oxygen free radicals. Among them, vitamin E is the major chain-breaking lipophilic antioxidant in tissues and plasma; the most biologically active form is \(\alpha\)-tocopherol. The results of human studies of the potential antiatherogenic role of vitamin E are still controversial. Some of them provide evidence of an association between a high intake of vitamin E and a lower risk of coronary heart disease [3] or show an inverse correlation between plasma vitamin E (E-pl) and a cardiovascular disease [4, 5]. In contrast, several studies found no direct associations between E-pl and mortality from coronary heart disease [6, 7]. The heterogeneity of the populations recruited for a variety of cardiovascular risk factors could be responsible for the controversial results reported from in vivo studies. Otherwise, the value of E-pl concentration alone as an index of vitamin E status is uncertain. Some authors suggested that the tocopherol of red blood cells (RBCs) or platelets associated with the tocopherol-to-lipid ratio of plasma could be more meaningful to evaluate the vitamin E status in humans [8, 9], but very few studies reported such measurements.

Thus, the present study was designed to assess vitamin E status [not only plasma, but also RBC vitamin E (E-RBC) concentrations] in asymptomatic hypercholesterolemic (HC) men. We investigated also the susceptibility of RBCs to an oxidative stress by determining the extent of hemolysis induced by a water-soluble azo compound.

Subjects and Methods

Subjects were obtained from an ongoing risk factor screening program conducted at their workplace for employees of several companies within the Paris, France, area by a group of occupational health physicians (PCVMETRA Group: Prévention Cardiovasculaire en Médecine du Travail) [10, 11]. After their consent was obtained, 57 men with a total cholesterol (TC) concentration >6.2 mmol/L (240 mg/dL) entered into the HC group of this study, and were compared with a normocholesterolemic group (NC) of 56 men. All subjects were free of

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1 Laboratoire de Biochimie and 2 Centre de Médecine Préventive Cardiovasculaire, Hôpital Broussais, 96 rue Didot, 75674 Paris Cedex 14, France.
2 Laboratoire de Biochimie Appliquée, Faculté des Sciences pharmaceutiques et biologiques, 92296 Châtenay-Malabry, France.
3 Laboratoire de Biochimie, Hôpital Broussais, 96 rue Didot, 75674 Paris Cedex 14, France. Fax 33 (0) 1 45 41 35 13.

Address correspondence to this author at: Laboratoire de Biochimie, Hôpital Broussais, 96 rue Didot, 75674 Paris Cedex 14, France. Fax 33 (0) 1 45 41 35 13.

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4 Nonstandard abbreviations: RBC, red blood cell; HC, hypercholesterolemic; NC, normocholesterolemic; TC, total cholesterol; TG, triglycerides; HDL-C, LDL-C, high-, low-density lipoprotein cholesterol; BHT, butylated hydroxytoluene; E-pl, plasma vitamin E; E-RBC, red blood cell vitamin E; AAPH, 2,2’-azo bis-(2-amidinopropane) dihydrochloride; LT, lag time; and T50, time required to achieve 50% hemolysis.
Distilled water containing 2 suspensions was made up to about 50% with addition of 0.5%pyrogallols as antioxidant agent. The final hematocrit 10 min at 4°C to separate plasma and RBCs. RBCs were expressed in mmol/L of plasma.

LDL and VLDL by a phosphotungstic acid-MgCl₂ reagent [14], which is accurate for TG concentrations <4.5 mmol/L: LDL-C = TC - HDL-C - (TG/2.2). The results were expressed in mmol/L of packed cells.

Blood collected into EDTA was centrifuged at 2000g for 10 min at 4°C to separate plasma and RBCs. RBCs were washed three times with NaCl solution (9 g/L) containing AAPH (200 mmol/L). Suspensions were incubated at 37 °C for 4 h under aerobic conditions and agitated gently throughout. The AAPH solution was incubated for 1 h at 37 °C and the RBC suspensions were incubated for 5 min at 37 °C before mixing. Aliquots were obtained at times ranging from 0 (corresponding to the time of mixing RBC suspensions with AAPH solution) to 240 min. Samples (50 μL) were diluted in 2 mL of NaCl (9 g/L) and centrifuged. The extent of hemolysis was measured spectrophotometrically at 540 nm, by comparing the extracellular hemoglobin content of the aliquots with that of a fully hemolyzed reference sample, which was prepared in the same way except that the AAPH solution was replaced by distilled water. Percentage of hemolysis was measured according to the equation: % hemolysis = A/B × 100, where A is absorbance of the sample aliquot at 540 nm and B is absorbance of the fully hemolyzed reference at 540 nm.

Results are expressed as mean ± SD. The statistical analysis was performed on an Apple Macintosh computer with the use of Statview (Abacus Concepts, Berkeley, CA). The Student’s t-test was used to compare the HC and the NC subjects. Data were analyzed in a univariate regression analysis. For all analyses, P <0.05 was considered significant.

Results

There were no statistical differences between the HC and the NC groups in age (HC = 47 ± 8 vs NC = 45 ± 10 years), systolic blood pressure (HC = 135 ± 16 vs NC = 140 ± 19 mmHg), diastolic blood pressure (HC = 86 ± 11 vs NC = 89 ± 14 mmHg), and proportion of smokers (HC = 29.8% vs NC = 33.9%). Compared with the control group, HC subjects had by definition higher TC (7.16 ± 0.75 vs 5.23 ± 0.64 mmol/L, P <0.0001) and higher LDL-C (5.18 ± 0.76 vs 3.50 ± 0.56 mmol/L, P <0.0001), but also had higher TG (1.35 ± 0.44 vs 0.98 ± 0.46 mmol/L, P <0.0001), although hypertriglyceridemic subjects (TG >2 mmol/L) were excluded. No differences existed between the two groups in HDL-C (1.31 ± 0.28 vs 1.28 ± 0.27 mmol/L).

As shown in Table 1, significantly higher E-pl was found in the HC group compared with the NC group, when the vitamin concentration was expressed in μmol/L of plasma. In agreement with previous reports [17, 18], we found a significant positive correlation between E-pl and TC (r = 0.59; P <0.0001), suggesting that plasma lipid concentration passively influences E-pl concentration. This correlation justified the recommendation that plasma lipid concentration be measured before antioxidant therapy.

Table 1. Comparison of total E-pl concentrations between HC and NC men.

<table>
<thead>
<tr>
<th></th>
<th>HC (n = 57)</th>
<th>NC (n = 56)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>E-pl (μmol/L)</td>
<td>42.49 ± 10.02</td>
<td>33.06 ± 7.48</td>
<td>0.0001</td>
</tr>
<tr>
<td>E-pl (μmol/mmol TC+TG)</td>
<td>5.00 ± 1.05</td>
<td>5.33 ± 0.98</td>
<td>NS</td>
</tr>
</tbody>
</table>

Results are expressed as mean ± SD.
tocopherol concentrations should be expressed relative to plasma lipid concentrations [17, 18]. Therefore, E-pl expressed relative to both TC and TG was not different between the two groups (Table 1).

To evaluate more accurately the vitamin E status associated with hypercholesterolemia, we also determined the E-RBC. This parameter was significantly lower in HC than in NC men (3.27 ± 0.70 vs 3.78 ± 1.10 μmol/L, P < 0.004), as shown in Fig. 1. Since there were smokers in the two populations, we verified that there was no statistical difference in the vitamin E content between smokers and nonsmokers in the HC group (3.42 ± 0.71 vs 3.20 ± 0.69 μmol/L) and in the NC group (3.69 ± 1.07 vs 3.63 ± 1.08 μmol/L). E-RBC was negatively correlated to TC concentrations (r = 0.33; P < 0.0005) and to LDL-C concentrations (r = 0.36; P < 0.0001).

To verify if the low E-RBC of HC men had consequences on RBC oxidation, we further studied the susceptibility of RBCs to an oxidative stress in an additional group of 51 subjects (27 HC and 24 NC men). The clinical and biological characteristics and the vitamin E status of these subjects were the same as those described above (data not shown). An example of the time-dependent rate of AAPH-induced RBC hemolysis is shown in Fig. 2. From the obtained sigmoid curves, we can calculate two quantitative parameters: (a) the lag time (LT, min), defined as the intercept between the linear least-square slope of the curve with the axis of incubation time, which reflects the capacity of the cell to buffer peroxyl radicals; and (b) the time required to achieve 50% hemolysis (T50, min). These two parameters were significantly decreased in HC men compared with NC subjects (LT = 97.8 ± 20.15 min and T50 = 124.4 ± 21.6 min) and with nonsmokers in the HC group (LT = 101.94 ± 13.15 min and T50 = 137.36 ± 17.95 min) and in the NC group (LT = 115.23 ± 16.81 min and T50 = 112.36 ± 18.52 min) and in the NC group (LT = 115.23 ± 16.81 min and T50 = 112.36 ± 18.52 min). The E-RBC concentration was positively correlated with LT (r = 0.37, P < 0.008) and with T50 (r = 0.30, P < 0.03).

Discussion

To clarify the potential antiatherogenic contribution of vitamin E, we have determined the vitamin E status of subjects free from any symptomatic cardiovascular diseases but with a well-known cardiovascular risk factor, hypercholesterolemia, compared with NC men. We did not find any statistical differences between the two groups in the E-pl concentrations when expressed relative to plasma lipid concentrations. In contrast, an original result of the present study relates the lower E-RBC content in HC men, despite their normal plasma tocopherol concentrations. This result demonstrates that the plasma α-tocopherol-to-lipid ratio is not a suitable predictor of cell vitamin E status, which could explain in part the discrepancy in results obtained between different human studies of the antiatherogenic role of vitamin E [3–7]. Moreover, with lipoproteins being the physiological transporters of vitamin E in plasma, this result could reflect a disturbance in the transfer of vitamin E between lipoproteins and RBCs and more generally tissues. Such an abnormal transfer of α-tocopherol from plasma to RBCs has been previously shown in chronic renal failure.
and in liver cirrhosis [19], but, to our knowledge, never in hypercholesterolemia. The hypothesis of an impaired transfer of vitamin E between lipoproteins and cells in hypercholesterolemia is indirectly sustained by the negative correlations obtained between E-RBC and concentrations of TC or LDL-C in this study. Furthermore, adipose tissue is one of the major stores oftocopherol in the body and the tocopherol efflux from this tissue could be important to maintain plasma and tissue concentrations during vitamin E deficiency (for review see ref. 20). Therefore adipose tissue tocopherol concentrations have been used as indicators of vitamin E status in patients [21–23]. Some studies have shown that in vitamin E-deficient patients there are mechanisms for the mobilization of tocopherol from adipose tissue [21, 22] and that α-tocopherol adipose tissue may be more readily available than previously thought. It would be of interest to measure the adipocyte vitamin E content in HC subjects to establish if the storage of vitamin E is also altered, in this pathology, because of putative impaired transfer.

To assess whether a decrease of E-RBC concentrations of HC subjects has a physiological consequence, we compared the susceptibility to peroxidation of RBCs from HC and NC men. Our results showed a significantly increased susceptibility of RBCs of HC men to AA-PH-induced oxidation. Moreover, we observed a direct correlation between the E-RBC concentration and susceptibility of cells to a peroxidative stress. Previous studies have shown that vitamin E has a protective effect on the RBC membrane against peroxidation and hemolysis induced by an azo compound. Some of these studies have been performed on isolated RBCs from rats [16] or humans [24], and showed that the addition of an excess of vitamin E in vitro reduced the susceptibility to a peroxidative stress. Other studies carried out on RBCs from vitamin E-deficient or -supplemented rabbits [25], rats [26], or humans [27] led to the same conclusion. By contrast with these studies, which modified experimentally the tocopherol RBC content, our results emphasize the essential role of vitamin E under physiopathological circumstances. Although the decrease of vitamin E in RBC of HC men is weak and does not correspond to a real deficiency, it is sufficient enough to impair the response to an oxidative stress. In addition, it is noteworthy that these results obtained in asymptomatic subjects are in good agreement with the hypothesis of Kritchevsky et al. [28], suggesting that antioxidant substances are likely to exert their effects at the earliest stages of the atherogenic process. Thus, in hypercholesterolemia, the evaluation of antioxidants might be taken into account as a risk marker with respect to the development of preclinical disease.

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