Antioxidant and prooxidant activity of \( \alpha \)-tocopherol in human plasma and low density lipoprotein

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Abstract \( \alpha \)-Tocopherol is a classical lipophilic antioxidant well known as a scavenger of free radicals in a hydrophobic milieu. However, it can develop both anti- and prooxidant activity in isolated low density lipoprotein (LDL). It is unknown how these activities are balanced in vivo in human plasma. We studied oxidation of plasma and LDL isolated from healthy donors or from a patient with familial isolated vitamin E deficiency and supplemented with \( \alpha \)-tocopherol in vivo or in vitro. We found that \( \alpha \)-tocopherol supplementation decreased plasma and LDL oxidizability under strong oxidative conditions when oxidation was initiated by high amounts of \( \text{Cu}^{2+} \) or 2,2'-azobis(2-amidinopropane) hydrochloride (AAPH). The effect was independent of the presence of ascorbate in the samples. Under conditions of mild oxidation by low amounts of \( \text{Cu}^{2+} \) or AAPH, \( \alpha \)-tocopherol supplementation decreased plasma oxidizability only in the presence of physiological amounts of ascorbate. A prooxidant effect of \( \alpha \)-tocopherol was found under mild oxidative conditions in highly diluted (150-fold) plasma and in isolated LDL. These results indicate that the level of oxidative stress and concentration of co-antioxidants, such as ascorbate, capable of regenerating \( \alpha \)-tocopherol in the oxidizing lipoprotein particle, appear to represent major factors determining \( \alpha \)-tocopherol activity towards oxidation both in human plasma and LDL. In vivo, in the presence of high concentrations of co-antioxidants and under mild oxidative conditions, \( \alpha \)-tocopherol should normally behave as an antioxidant. This antioxidant activity is also expected to prevail under strong oxidative conditions independently of the presence of co-antioxidants but it may evolve into prooxidant, when the co-antioxidants are exhausted under conditions of mild oxidation. It remains to be shown whether such a transformation is physiologically relevant and can occur in vivo.

Supplementary key words \( \alpha \)-tocopherol • ascorbate • lipid peroxidation • blood plasma • low density lipoprotein • atherosclerosis

There is increasing evidence that suggests that the oxidation of low density lipoprotein (LDL) in the arterial wall is an important step in the early development of atherosclerosis (1, 2). The exact mechanism by which LDL undergoes oxidation in vivo is not yet fully understood but there is little doubt that it involves free radical oxidation of LDL lipids (3, 4). Preventing the oxidation of LDL using antioxidants is widely discussed at present as a promising antiatherosclerotic therapy (2, 5). This implies that LDL-associated lipid-soluble antioxidants able to efficiently protect the lipoprotein against oxidation may represent important antiatherosclerotic agents.

\( \alpha \)-Tocopherol is a classical lipid-soluble antioxidant well known as a scavenger of free radicals in a hydrophobic milieu (6). It is well established that \( \alpha \)-tocopherol is, on a molar base, the major antioxidant in LDL (3, 4). All other antioxidants (\( \gamma \)-tocopherol, carotenoids, ubiquinol-10) are present in LDL in much smaller amounts. Inhibiting LDL oxidation by increasing its \( \alpha \)-tocopherol content would be a very attractive way to prevent atherogenesis. Data on the antioxidant function of \( \alpha \)-tocopherol in lipoproteins are, however, contradictory. The rate of LDL oxidation is generally expected to be low in the presence and high in the absence of \( \alpha \)-tocopherol (lag-phase and propagation phase of the oxidation, respectively) (3). However, LDL oxidizability by \( \text{Cu}^{2+} \) has only been found to correlate negatively with LDL \( \alpha \)-tocopherol when the lipoprotein is enriched with this antioxidant (7–10) and when conditions of a relatively strong oxidative stress are used (11). No significant correlation between \( \alpha \)-tocopherol content and susceptibility to oxidation in native LDL unsupplemented with antioxidants has been found under such oxidative conditions (7, 8, 12–18). When mild oxidative conditions

Abbreviations: AAPH, 2,2'-azobis(2-amidinopropane) hydrochloride; DMSO, dimethyl sulfoxide; EDTA, ethylenediaminetetraacetic acid; FIVE, familial isolated vitamin E deficiency; HPLC, high performance liquid chromatography; LDL, low density lipoprotein; PBS, phosphate-buffered saline; PUFA, polyunsaturated fatty acid.

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TABLE 1. Antioxidant and PUFA content of plasma samples obtained from donors supplemented with vitamin E

<table>
<thead>
<tr>
<th>Donor, Sample</th>
<th>α-Tocopherol</th>
<th>Ubiquinol-10</th>
<th>PUFAs</th>
<th>µM</th>
</tr>
</thead>
<tbody>
<tr>
<td>FIVE patient</td>
<td>Day 0</td>
<td>3.09</td>
<td>1.00</td>
<td>4273</td>
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<tr>
<td></td>
<td>Day 1</td>
<td>15.5</td>
<td>0.94</td>
<td>4140</td>
</tr>
<tr>
<td></td>
<td>Day 2</td>
<td>32.6</td>
<td>1.02</td>
<td>3518</td>
</tr>
<tr>
<td></td>
<td>Day 3</td>
<td>65.7</td>
<td>0.98</td>
<td>3457</td>
</tr>
<tr>
<td>Healthy donor A</td>
<td>0 h</td>
<td>16.2</td>
<td>n.d.</td>
<td>2846</td>
</tr>
<tr>
<td></td>
<td>2 h</td>
<td>20.0</td>
<td>n.d.</td>
<td>3184</td>
</tr>
<tr>
<td></td>
<td>4 h</td>
<td>27.4</td>
<td>n.d.</td>
<td>3134</td>
</tr>
</tbody>
</table>

Plasma samples were obtained from a FIVE patient on 4 different days before and during his supplementation with vitamin E and from healthy donor A immediately before and 2 and 4 h after his single-dose supplementation with 1500 IU vitamin E; n.d., not determined.

are used to oxidize LDL, increasing LDL \( \alpha \)-tocopherol has paradoxically been shown to increase LDL oxidizability (11, 19). This effect has been ascribed to the prooxidant activity of \( \alpha \)-tocopheroxyl radical formed at early oxidation stages. This radical, if present in the LDL particle for a sufficient time, i.e., at low free radical fluxes, can directly oxidize LDL polyunsaturated fatty acids (PUFAs) (11, 19). Nevertheless, the potentially harmful \( \alpha \)-tocopheroxyl radical can be efficiently eliminated from the LDL particle by so-called co-antioxidants (20, 21), such as ascorbate (21) or bilirubin (20), capable of recycling it back into \( \alpha \)-tocopherol. Such a recycling is expected to inhibit the prooxidant activity of \( \alpha \)-tocopherol. These findings indicate that \( \alpha \)-tocopherol can develop both anti- and prooxidant activity in isolated LDL, depending on oxidative conditions and presence of co-antioxidants. Our recent data suggest that a net effect of \( \alpha \)-tocopherol on LDL oxidation is determined by a subtle balance of its anti- and prooxidant effects (16, 18). It is unknown, however, how these effects are balanced in vivo in human plasma.

In the present study we aimed to characterize a net activity of \( \alpha \)-tocopherol towards oxidation in human plasma and LDL. We oxidized plasma and LDL containing different amounts of \( \alpha \)-tocopherol under different oxidative conditions and in the presence of different amounts of ascorbate. Plasma and LDL \( \alpha \)-tocopherol content was varied by supplementing the donors (healthy normolipidemic subjects and a patient with familial isolated vitamin E deficiency (FIVE) (22–24)) or isolated plasma with \( \alpha \)-tocopherol. We found that all kinds of \( \alpha \)-tocopherol supplementation decreased plasma and LDL oxidizability under strong oxidative conditions independently of the presence of ascorbate in the samples. Under conditions of mild oxidation, \( \alpha \)-tocopherol supplementation decreased plasma oxidizability only in the presence of physiological amounts of ascorbate, suggesting that in vivo, in the presence of high concentrations of co-antioxidants, \( \alpha \)-tocopherol behaves as an antioxidant independently of the oxidative conditions.

MATERIALS AND METHODS

Chemicals

Chelex 100 resin (50–100 mesh) was obtained from Bio-Rad (Richmond, CA). All other chemicals and solvents were from Sigma (Deisenhofen, Germany) or Merck (Darmstadt, Germany). All reagents used for plasma and LDL oxidation were made up in Chelex-treated, double-distilled deionized water to minimize contamination with transition metal ions.

Plasma and LDL isolation

Plasma and LDL were obtained from a 27-year-old FIVE patient (22–24) and from several healthy normolipidemic donors not on a special antioxidant or fatty acid diet. To obtain plasma and LDL enriched with \( \alpha \)-tocopherol, supplementation of the donors with vitamin E in vivo and supplementation of the plasma with \( \alpha \)-tocopherol in vitro were used.

The FIVE patient and healthy donor A were given vitamin E. The vitamin was given as capsules containing generic \( \alpha \)-tocopherol acetate (La Roche, Basel, Switzerland). The FIVE patient was homozygous for defect in the \( \alpha \)-tocopherol transfer protein gene (24) and re-
Fig. 1. Accumulation of conjugated dienes (O) and oxycholesterols (7-hydroxycholesterol (●), 20-hydroxycholesterol (▲) and 25-hydroxycholesterol (■); A) and consumption of PUFAs (linoleic (●) and arachidonic (▲) acids; B) in heparin plasma of a healthy donor A. The plasma was diluted 150-fold with PBS containing 0.16 M NaCl and oxidized by 50 µM Cu²⁺ at 37°C in a spectrophotometrical cuvette to measure conjugated dienes or in a water bath to measure oxycholesterols and PUFAs. The results shown are means of two independent experiments.

quired regular vitamin E supplements (1800 mg daily). He was completely withdrawn from his oral vitamin E supplementation 5 days before the first blood sample was taken (day 0). Then, he was resupplemented with increasing amounts of vitamin E (400, 1200, and 1800 mg on the first, second, and third days of the resupplementation). Blood was taken from the patient for the first 3 days after resuming the supplementation (days 1, 2, and 3). All patient's blood samples were taken into heparin- or ethylenediaminetetraacetic acid (EDTA)-containing tubes (Sarstedt, Nürnberg, Germany) after an overnight fast. To control for changes in plasma α-tocopherol and fatty acid levels in the course of the supplementation, the latter was repeated (see Results). Fasted donor A was supplemented with a single dose of 1500 IU vitamin E and the blood was taken immediately before and 2 and 4 h after supplementation. Plasma was obtained by centrifugation of the blood at 4°C for 10 min.

In separate experiments, plasma of healthy donors A (unsupplemented with vitamin E) and B was supplemented with α-tocopherol in vitro. The blood was taken after an overnight fast into heparin- or EDTA-containing tubes. The plasma was incubated for 3 h at 4°C with α-tocopherol, added as a solution in dimethyl sulfoxide (DMSO) to a final concentration of 167 and 333 µM (25). Control incubations contained a corresponding amount of dimethylsulfoxide (1% v/v) which was the same in all plasma samples used. All plasma samples were frozen at -80°C under nitrogen immediately after isolation and stored under these conditions for no longer than 2 months.

LDL was isolated by density gradient ultracentrifugation of the EDTA plasma for 20 h at 4°C (26). All density gradient solutions contained 1.5 mM EDTA. The resulting EDTA-containing LDL suspensions were stored at 4°C under nitrogen in the dark and used for experiments within 24 h of isolating the LDL.

Characterization of plasma and LDL chemical composition

Plasma and LDL content of α-tocopherol and ubiquinol-10 were quantified by reversed-phase high performance liquid chromatography (HPLC) with electrochemical detection using SuperPack Pep-S RF C₇/C₁₈ HPLC-column (250 x 4.0 mm i.d., 5 µm particle size, Pharmacia, Fine Chemicals, Uppsala, Sweden) as described elsewhere (27). γ-Tocotrienol, ubiquinol-9, and ubiquinone-7 were used as internal standards. In some experiments, plasma content of α-tocopherol was determined by reversed-phase HPLC with fluorescence detection and δ-tocopherol as an internal standard. Briefly, 100 µl plasma or 100 µl LDL (0.85 mg total cholesterol/ml) was mixed with 100 µl ethanol containing the necessary amount of δ-tocopherol. After adding 500 µl hexane, the mixture was vortexed and centrifuged and the hexane extract was collected and evaporated under nitrogen. The residue was dissolved in 100 µl methanol and subjected to HPLC with a fluorescent detector (Shimadzu RF-535 Fluorescence HPLC Monitor, Shimadzu, Japan) adjusted at 294 nm excitation and 325 nm emission. As a mobile phase, methanol–water 98:2 (v/v) mixture at a flow rate of 2.0 ml/min was used.

Fatty acid composition of plasma and LDL was characterized by capillary gas chromatography (28). One hundred µl plasma or 100 µl LDL (0.85 mg total cholesterol/ml) was mixed with 400 µl toluene and 1.6 ml methanol. Tricosanoic acid and t-butylhydroxytoluene solutions in ethanol were added as an internal standard and antioxidant, respectively. After vortexing, 200 µl acetylchloride was added. The reaction mixture was incubated for 1 h at 100°C, cooled, mixed with 5 ml of
6% Na2CO3, and centrifuged for 10 min at 3000 rpm. Two hundred µl toluene phase was removed, evaporated under nitrogen at room temperature, and dissolved in 20 µl hexane. One µl of the hexane extract was injected into a Hewlett-Packard 5890 Series II gas chromatograph (Hewlett-Packard, Palo Alto, CA) equipped with a flame ionization detector. Fatty acid methyl esters were chromatographed on a 30 m HP-5MS column (Hewlett-Packard, Palo Alto, CA) with an internal diameter of 0.25 mm. Helium was used as carrier gas at a flow rate of 1.2 ml/min. The injection port temperature was 250°C and the detector was 300°C. The column temperature was held at 170°C for 2 min, increased to 220°C at a rate of 4°C/min, held at 220°C for 4 min, and increased to 310°C at a rate of 15°C/min. The peak quantification was based on peak area comparison with the internal standard. Plasma and LDL content of PU-FAs were calculated as a sum of their content of linoleic, linolenic, γ-linolenic, eicosadienoic, eicosatrienoic, arachidonic, eicosapentaenoic, docosadienoic, docosatetraenoic, docosapentaenoic, and docosahexaenoic acids.

LDL total cholesterol was determined by a commercially available enzymatic test (Boehringer Mannheim, Mannheim, Germany). To recalculate the values obtained into mol/mol LDL, LDL molecular mass of 2.5 MDa and its total cholesterol content of 31.6% of the total weight were used (3, 29).

**Plasma and LDL oxidation**

Cu²⁺ and 2,2’-azobis-(2-amidinopropane) hydrochloride (AAPH) were used to oxidize plasma and LDL in our study. Plasma was oxidized by Cu²⁺ as described by Regnström et al. (30). Heparin plasma sample (20 µl) was diluted with 2950 µl phosphate-buffered saline (PBS) containing 0.16 M NaCl. The oxidation was then started by adding 30 µl of 50 µM or 5 mM CuSO₄ solution, corresponding to the mild and strong oxidative conditions, respectively. To oxidize plasma by AAPH, 20 µl of the heparin plasma were diluted with 2880 µl PBS containing 0.16 M NaCl and 100 µl of 10 mM AAPH solution were added. The oxidation was performed in a spectrophotometrical cuvette at 37°C to register an accumulation of conjugated dienes in the samples at 234 nm. Plasma dilution (150-fold in both cases) was necessary to provide absorbance low enough to be reliably measured (30, 31). To provide a direct measure of lipid peroxidation in the samples, plasma was oxidized at the same dilution in a 37°C water bath and consumption of PUFA and accumulation of oxy-cholesterols were measured (see below). To investigate

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**Fig. 2.** Accumulation of conjugated dienes in heparin plasma of a FIVE patient supplemented with vitamin E. The plasma was obtained on days 0 (C), 1 (D), and 3 (E) of the supplementation, diluted 150-fold with PBS containing 0.16 M NaCl and oxidized in a spectrophotometrical cuvette at 37°C by Cu²⁺ (A, B) or AAPH (C). Cu²⁺ concentration, 50 µM (A) or 0.5 µM (B); AAPH concentration, 333 µM (C).
an effect of such a high dilution on oxidation, the plasma was diluted 2-fold with PBS containing 0.16 M NaCl and oxidized at 37°C in the presence of 25 mM AAPH. Accumulation of oxycholesterols and an increase of fluorescence at 360/430 nm were used to characterize the oxidation under these conditions (see below). The final AAPH concentration calculated per plasma volume was the same for both 150-fold and 2-fold dilutions (50 μM).

To oxidize LDL, EDTA and potassium bromide were removed from the LDL suspensions by gel filtration on Sephadex PD-10 columns (Sephadex G-25M, Pharmacia Fine Chemicals, Uppsala, Sweden) immediately before oxidation. The EDTA-free LDL suspensions were diluted with PBS to 0.12 mg total cholesterol/ml (approximately 0.14 μM LDL). Oxidation by Cu²⁺ was performed at 30°C in a spectrophotometrical cuvette at a Cu²⁺ concentration of 0.01, 0.1, or 3.0 μM. Oxidation by AAPH was performed at 37°C in a spectrophotometrical cuvette at an AAPH concentration of 70 μM. In some experiments, a small volume of freshly prepared aqueous solution of ascorbate was added to the plasma or LDL immediately before oxidation.

Characterization of the level of plasma and LDL oxidation

To characterize the level of plasma oxidation, accumulation of conjugated dienes and oxycholesterols, consumption of α-tocopherol and fatty acids as well as fluorescence of the samples were measured. Accumulation of conjugated dienes was evaluated at 234 nm as described by Regnström et al. (30). Time-course of conjugated diene accumulation under strong oxidative conditions was characterized by a distinct lag-phase followed by a propagation phase (30, 31). Freezing and thawing of plasma had no significant influence on its oxidation course as judged by the duration of the oxidation phases measured in freshly obtained samples and in plasma samples frozen for 24 h from six healthy donors (lag-phase 292 ± 132 and 311 ± 115 min, propagation phase 125 ± 22 and 107 ± 8 min, total lag-phase + propagation phase 417 ± 142 and 418 ± 113 min, respectively).

Accumulation of oxycholesterols was measured using capillary gas chromatography (32, 33). Two hundred μl plasma (diluted 2-fold) was mixed with 900 μl chloro-
Fig. 4. Influence of ascorbate on the accumulation of conjugated dienes in heparin plasma of a FIVE patient supplemented with vitamin E. The plasma was obtained on days 0 (●), 1 (○), and 3 (▲) of the supplementation, diluted 150-fold with PBS containing 0.16 M NaCl and oxidized in a spectrophotometrical cuvette at 37°C by 333 μM AAPH. Ascorbate was added to the plasma immediately before oxidation to the final concentration of 100 μM (A) or 10 mM (B).

form-methanol 2:1 (v/v) mixture. 5α-Cholesterol solution in chloroform-methanol 2:1 (v/v) mixture and t-butylhydroxytoluene solution in ethanol were added as an internal standard and antioxidant, respectively. Chloroform plasma extract was evaporated under nitrogen and dissolved in 500 μL of diethyl ether-methanol 1:1 (v/v) mixture containing sodium borohydride (20 mg/ml) to reduce hydroperoxides to hydroxides. The reduction was performed for 30 min at room temperature. Diethyl ether phase was collected, evaporated under nitrogen, and the residue was dissolved in 250 μL toluene. After adding 500 μL of 0.5 M sodium methoxide solution in methanol, the reaction mixture was incubated for 15 min at 50°C, cooled, mixed with 1 mL of 2.5% acetic acid, and extracted with hexane. The hexane phase was evaporated under nitrogen and the residue was dissolved in 80 μL acetone and 20 μL bis(trimethylsilyl)trifluoroacetamide containing 1% trimethylchlorosilane. The reaction mixture was incubated for 45 min at room temperature, evaporated under nitrogen, and the residue was dissolved in 20 μL toluene. One μL of the toluene solution was injected into the same gas chromatograph that was used to measure plasma fatty acids. Plasma content of oxycholesterols was calculated as the sum of its content of 7β-hydroxy-, 20-hydroxy-, and 25-hydroxycholesterols, 7-ketocholesterol and cholestadien-7-one. Individual oxycholesterols were identified by gas chromatography-mass spectrometry using a Hewlett-Packard MS 5972 mass selective detector (Hewlett-Packard, Palo Alto, CA) (32, 33).

α-Tocopherol and PUFAs in oxidized plasma samples were quantified chromatographically as described above. Plasma fluorescence was measured with an excitation at 360 nm and an emission at 430 nm (34, 35). Slit width of 5 nm was used for both emission and excitation slits. To prove whether the plasma fluorescence at 360/430 nm was indicative of the lipoprotein oxidation, non-oxidized and oxidized (for 24 h at 37°C in the presence of 7.5 mM Cu²⁺) plasma from healthy donor A was ultracentrifuged to isolate lipoproteins as described above and the fluorescence of lipoprotein fractions was measured at 360/430 nm.

LDL oxidation was evaluated as an accumulation of conjugated dienes in the samples. Conjugated dienes were measured according to Puhl, Waeg, and Esterbauer (29) and Esterbauer et al. (36) by continuous registration of sample absorption at 234 nm. The duration of the lag-phase of diene accumulation was calculated from the curves thus obtained (16, 29, 36).

Statistical analysis

Unless specified, the data shown represent typical results obtained in four independent experiments. Significance of the differences in the oxidation rates between α-tocopherol-supplemented and non-supplemented samples was evaluated using the Friedman two-way analysis of variance (ANOVA).

RESULTS

Antioxidant and prooxidant activity of α-tocopherol in human plasma

Withdrawal of the FIVE patient from his regular vitamin E supplementation for 5 days resulted in a dramatic decrease in plasma α-tocopherol to a hardly detectable level (3.09 μM) (Table 1). Resupplementation of the patient with increasing amounts of vitamin E for 3 days restored the plasma concentration to a high level. Marked increase of plasma α-tocopherol was not accompanied by any consistent change in the level of ubiquinol-10, an important antioxidant influencing lipoprotein oxidizability (16, 18, 37, 38). Plasma PUFAs, another...
important determinant of lipoprotein oxidizability (16, 39), were decreased after α-tocopherol supplementation. However, the latter effect was inconsistent and was not found when the supplementation of the patient with vitamin E was repeated (data not shown). Single supplementation of healthy donor A with a high dose of α-tocopherol (1500 IU) also increased the plasma level considerably (measured 2-fold with PBS containing 0.16 M NaCl and oxidized at 37°C in the presence of 25 mM AAPH. The results shown are means of two independent experiments.

Incubation of the plasma with Cu²⁺ resulted in an increase in its absorbance at 234 nm (Fig. 1). Such an increase is related to the accumulation of conjugated dienes in the samples (30, 36) and has been shown to correlate with other indices of plasma lipid peroxidation (30). Plasma oxidation measured as an increase in absorbance at 234 nm has also been found to reflect oxidation of plasma lipoproteins (30, 31). We found that the time-course of conjugated diene accumulation paralleled those of oxycholesterol formation (Fig. 1, A) and PUFA consumption (Fig. 1, B) directly measured in the same samples by gas chromatography. We also found that the rates of conjugated diene accumulation measured in 23 plasma samples negatively correlated with initial plasma concentrations of different antioxidants, such as α-tocopherol, ubiquinol-10, ascorbate, bilirubin, and albumin (Spranger, T., U. Beisiegel, and A. Kontush, unpublished data), and that the in vitro supplementation of plasma with physiological amounts of these antioxidants resulted in a considerable delay of the oxidation under identical experimental conditions (Karten, B., A. Kontush, and U. Beisiegel, unpublished data). Taken together, these data justified using an increase in absorbance of oxidizing plasma at 234 nm as a measure of plasma lipoprotein oxidation.

Oxidation of plasma samples with different α-tocopherol content revealed that increasing α-tocopherol concentration in the FIVE patient's plasma decreased the rate of conjugated diene accumulation in the samples (Fig. 2, A). Oxidation rate was significantly decreased both within the lag-phase and propagation phase of conjugated diene accumulation (n = 4, P < 0.05). The antioxidant activity of α-tocopherol was seen in highly diluted (150-fold) plasma under strong oxidative conditions (in the presence of 50 μM Cu²⁺). This activity was independent of changing plasma levels of PUFA and was found irrespectively of whether plasma PUFA concentration decreased or not in the course of the vitamin E supplementation (data not shown). Adding ascorbate (100 μM) to the plasma samples also did not qualitatively change the dependence of plasma oxidizability on α-tocopherol concentration (data not shown).

Using milder oxidative conditions reversed the dependence of the oxidizability of highly diluted plasma on its α-tocopherol concentration. Plasma samples with higher α-tocopherol level were oxidized faster than those with lower α-tocopherol in the presence of 0.5 μM Cu²⁺ (n = 4, P < 0.05; Fig. 2, B). Enrichment with α-tocopherol also increased oxidizability of the patient's plasma when it was diluted 150-fold and oxidized in the presence of 333 μM AAPH (n = 4, P < 0.05; Fig. 2, C). Such oxidative conditions (50 mM AAPH calculated per plasma volume) are generally considered to be strong enough to lead to an antioxidant activity of α-tocopherol (11, 40, 41). The prooxidant activity found in our study suggests that the actual level of oxidative stress applied to plasma under these conditions was greatly diminished due to the high dilution of the reaction mixture and that these conditions should be considered as mild.

Similar dependences of the oxidizability of highly diluted plasma on its α-tocopherol concentration were observed, when plasma samples from healthy donors A and B were enriched with α-tocopherol in vitro and oxidized under strong (50 μM Cu²⁺; Fig. 3, A) and mild (0.5 μM Cu²⁺, 333 μM AAPH; Fig. 3, B, C) oxidative conditions (n = 5, P < 0.05; Fig. 3). A prooxidant activity of α-tocopherol was also found under mild oxidative conditions in highly diluted plasma from healthy donor A supplemented with vitamin E in vivo (data not shown). Slightly increased concentration of plasma PUFA could also contribute to the latter effect (Table 1).

Supplementing the FIVE patient's plasma with a physiological amount of ascorbate (100 μM) did not prevent the prooxidant activity developed by α-tocopherol in highly diluted plasma under mild oxidative conditions.
supplemented with vitamin E. The plasma was obtained from heparin plasma of a patient and of healthy donor A (B). The plasma was diluted 2-fold with PBS containing 0.16 M NaCl and oxidized at 37°C, resulting in the physiological final concentration of 1.24 ± 0.22 to 9.15 ± 0.66 for VLDL, from 0.29 ± 0.04 to 5.93 ± 1.54 for LDL, and from 0.22 ± 0.05 to 29.2 ± 3.5 arbitrary units/mg total cholesterol for HDL (means ± SD for four experiments). Vitamin E supplementation of the FIVE patient decreased the oxidizability of his plasma samples under strong oxidative conditions, when measured either as an accumulation of oxycholesterols or as an increase of fluorescence at 360/430 nm (Fig. 5). Vitamin E supplementation of both the FIVE patient and healthy donor A decreased the oxidizability of their plasma samples under identical oxidative conditions, when measured as an increase of fluorescence at 360/430 nm (n = 4, P < 0.05; Fig. 6). Additional supplementation of the plasma with a physiological amount of ascorbate (100 pM) did not significantly influence the vitamin E antioxidant activity under these oxidative conditions (data not shown). Increased plasma protection against oxidation provided by α-tocopherol was also seen when α-tocopherol consumption was measured. In accordance with previous findings, absolute concentration of α-tocopherol was consistently higher in the samples with higher initial α-tocopherol content at all time points until total α-tocopherol oxidation (Fig. 7). On the other hand, no consistent differences in the consumption of plasma PUFAs among samples with different α-tocopherol content were found (Fig. 7). This could be due to the fact that the oxidizability differences between samples were too small to be seen as differences in plasma PUFAs levels (accuracy of the determination ±7%, i.e., about ±200 μM PUFAs at their initial level about 3000 μM; see Fig. 7).

Antioxidant and prooxidant activity of α-tocopherol in human LDL.

Increasing the concentration of α-tocopherol in the FIVE patient's plasma paralleled the increasing LDL α-tocopherol content (Table 2). No consistent change in the level of LDL ubiquinol-10 and PUFAs during vitamin E supplementation was found. When the FIVE patient's LDL was oxidized under strong oxidative conditions (in the presence of 3.0 μM Cu²⁺), α-tocopherol revealed an antioxidant activity. This was seen as a prolonged lag-phase of conjugated diene accumulation (Table 2). Similar antioxidant action of α-tocopherol supplementation was also found in LDL isolated from healthy donor plasma supplemented with α-tocopherol in vitro (data not shown). α-Tocopherol retained its antioxidant activity...
with vitamin E (see Materials and Methods). The plasma was diluted with vitamin E. The plasma was obtained on days 0 (opened symbols) and 3 (closed symbols) of the repeated supplementation of the patient.

**DISCUSSION**

α-Tocopherol was found to develop both anti- and prooxidant activity in human plasma and isolated LDL, depending on oxidative conditions and presence of ascorbate in the samples. Antioxidant activity prevailed in plasma and LDL incubated under strong oxidative conditions (incubation of the diluted 150-fold and 2-fold plasma with 50 μM Cu²⁺ and 25 mM AAPH, respectively, and incubation of the diluted to 0.14 μM LDL with 3.0 μM Cu²⁺). Under such conditions, α-tocopherol behaved as an antioxidant independently of the presence of ascorbate in the samples, based on the observation that α-tocopherol supplementation reduced oxidation both in the plasma and in isolated LDL independently of their supplementation with different amounts of ascorbate.

The antioxidant activity of α-tocopherol was switched into prooxidant when milder conditions were used to initiate oxidation. Supplementation with α-tocopherol accelerated oxidation in plasma and LDL under mild oxidative conditions (incubation of the diluted 150-fold plasma with 0.5 μM Cu²⁺ or 333 μM AAPH and incubation of the diluted to 0.14 μM LDL with 0.1 or 0.01 μM Cu²⁺ or 70 μM AAPH). However, such a prooxidant effect of α-tocopherol was only found if the samples were virtually free of ascorbate (such as LDL subjected to the gel filtration on Sephadex columns) or if the final concentration of ascorbate in the samples was unphysiologically low (such as that in plasma diluted 150-fold). Adding ascorbate to a near-physiological final concentration restored the antioxidant activity of α-tocopherol also under mild oxidative conditions.

α-Tocopherol is a well-known lipid-soluble antioxidant able to scavenge free radicals in a hydrophobic milieu (6). The major mechanism of its antioxidative action includes inactivation of one radical (R⁻) by one molecule of α-tocopherol (TocH),

\[
\text{TocH} + \text{R}^- \rightarrow \text{RH} + \text{Toc}^+; \quad \text{Reaction 1)
\]

with a subsequent scavenging of a second radical by the α-tocopheroxyl radical (Toc⁺) formed:

\[
\text{Toc}^- + \text{R}^- \rightarrow \text{non-radical products}; \quad \text{Reaction 2)
\]

Resulting inactivation of two radicals per one molecule of α-tocopherol underlies the classical antioxidant activity of this compound. This mechanism is operative

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**Fig. 7.** Consumption of α-tocopherol (circles) and PUFAs (triangles) during oxidation of heparin plasma of a FIVE patient supplemented independent experiments.

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<table>
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<th>Time of oxidation, h</th>
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<th>mPm PUFAs</th>
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<tr>
<td>1</td>
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<td>8</td>
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α-Tocopherol was found to develop both anti- and prooxidant activity in human plasma and isolated LDL, depending on oxidative conditions and presence of ascorbate in the samples. Antioxidant activity prevailed in plasma and LDL incubated under strong oxidative conditions (incubation of the diluted 150-fold and 2-fold plasma with 50 μM Cu²⁺ and 25 mM AAPH, respectively, and incubation of the diluted to 0.14 μM LDL with 3.0 μM Cu²⁺). Under such conditions, α-tocopherol behaved as an antioxidant independently of the presence of ascorbate in the samples, based on the observation that α-tocopherol supplementation reduced oxidation both in the plasma and in isolated LDL independently of their supplementation with different amounts of ascorbate.

The antioxidant activity of α-tocopherol was switched into prooxidant when milder conditions were used to initiate oxidation. Supplementation with α-tocopherol accelerated oxidation in plasma and LDL under mild oxidative conditions (incubation of the diluted 150-fold plasma with 0.5 μM Cu²⁺ or 333 μM AAPH and incubation of the diluted to 0.14 μM LDL with 0.1 or 0.01 μM Cu²⁺ or 70 μM AAPH). However, such a prooxidant effect of α-tocopherol was only found if the samples were virtually free of ascorbate (such as LDL subjected to the gel filtration on Sephadex columns) or if the final concentration of ascorbate in the samples was unphysiologically low (such as that in plasma diluted 150-fold). Adding ascorbate to a near-physiological final concentration restored the antioxidant activity of α-tocopherol also under mild oxidative conditions.

α-Tocopherol is a well-known lipid-soluble antioxidant able to scavenge free radicals in a hydrophobic milieu (6). The major mechanism of its antioxidative action includes inactivation of one radical (R⁻) by one molecule of α-tocopherol (TocH),

\[
\text{TocH} + \text{R}^- \rightarrow \text{RH} + \text{Toc}^+; \quad \text{Reaction 1)
\]

with a subsequent scavenging of a second radical by the α-tocopheroxyl radical (Toc⁺) formed:

\[
\text{Toc}^- + \text{R}^- \rightarrow \text{non-radical products}; \quad \text{Reaction 2)
\]

Resulting inactivation of two radicals per one molecule of α-tocopherol underlies the classical antioxidant activity of this compound. This mechanism is operative
when free radicals are formed at a relatively high rate, i.e., under strong oxidative conditions (11, 21). It is likely responsible for the antioxidant activity of α-tocopherol found under strong oxidative conditions in our study. Antioxidant activity of α-tocopherol has been shown to evolve into prooxidant when mild oxidative conditions are used to oxidize isolated human LDL (11, 19, 21). This prooxidant activity is ascribed to the chain propagation by α-tocopheroxyl radical formed in reaction 1 at an early oxidation stage (11, 19). If no additional free radicals hit the LDL particle for a certain time and interacts with the α-tocopheroxyl radical (i.e., under mild oxidative conditions), the latter can directly oxidize LDL PUFA moieties:

\[
\text{Toc}^\cdot + \text{LH} \rightarrow \text{TocH} + \text{L}^\cdot, \quad \text{Reaction 3)
\]

where LH and L\text{ch} denote the PUFA moiety and its radical. This mechanism could underlie the prooxidant activity of α-tocopherol found towards oxidation of diluted 150-fold plasma and isolated LDL in the presence of AAPH (which provided a steady and mild flux of free radicals under our experimental conditions (11)). α-Tocopheroxyl radical can also be formed in the course of plasma and LDL oxidation by Cu\text{2+} via direct interaction between Cu\text{2+} and α-tocopherol (43, 44):

\[
\text{Cu}^{2+} + \text{TocH} \rightarrow \text{Cu}^+ + \text{Toc}^\cdot + \text{H}^+. \quad \text{Reaction 4)
\]

Reactions 3 and 4 could be responsible for the prooxidant activity of α-tocopherol found in our study in the presence of low amounts of Cu\text{2+}. This scheme of the α-tocopherol-mediated peroxidation (11, 21) suggests that the fate of α-tocopheroxyl radical is crucial for the net α-tocopherol activity towards oxidation. Rapid elimination of α-tocopheroxyl radical via reaction with another lipid radical leads to the net antioxidant activity of α-tocopherol. Prolonged residence of α-tocopheroxyl radical in the lipoprotein particle results in its slow reaction with lipoprotein PUFA’s and in the net prooxidant activity of α-tocopherol. It appears that α-tocopheroxyl radical must be efficiently eliminated from the lipoprotein particle to allow α-tocopherol to develop its antioxidant activity. Elimination of α-tocopheroxyl radical by recycling it directly back into α-tocopherol represents an important mechanism of action of a wide group of compounds called α-tocopherol co-antioxidants (21). Ascorbate (21) and bilirubin (20) seem to be physiologically the most important amongst them because of their high concentrations in human plasma (45). Reaction between ascorbate and α-tocopherol might be responsible for the restoration of the antioxidant activity of α-tocopherol under mild oxidative conditions in the presence of the former, as it was found in our study:

\[
\text{Toc}^\cdot + \text{AscH} \rightarrow \text{TocH} + \text{Asc}^\cdot, \quad \text{Reaction 5)
\]

where AscH and Asc\text{ch} denote ascorbate and its radical. This reaction has been shown to occur at a high rate in vitro under physiological conditions (46, 47). Our results indicate that the concentration of ascorbate in the reaction mixture must be high enough (within the physiological range) to provide an efficient elimination of α-tocopheroxyl radical via reaction (5).

Taken together, our data suggest that the level of oxidative stress and concentration of co-antioxidants, such as ascorbate, represent major factors governing α-tocopherol activity towards oxidation in human plasma and LDL. Increasing oxidative stress and/or co-antioxidant concentrations appear to promote an antioxidant activity of α-tocopherol. Decreasing oxidative stress encounters the α-tocopherol antioxidant activity and can reverse it into prooxidant. It seems, however, that decreasing co-antioxidant concentrations can only turn α-tocopherol into a prooxidant under mild oxidative conditions. Under strong oxidative conditions, α-tocopherol is expected to behave antioxidatively independently of the presence of ascorbate in the samples.

### Table 2. Antioxidant, PUFA content and oxidative resistance of LDL samples isolated from a FIVE patient supplemented with α-tocopherol in vivo

<table>
<thead>
<tr>
<th>Sample</th>
<th>α-Tocopherol</th>
<th>Ubiquinol-10</th>
<th>PUFAs</th>
<th>5.0 μM Cu</th>
<th>0.1 μM Cu</th>
<th>0.01 μM Cu</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 0</td>
<td>0.37</td>
<td>0.107</td>
<td>1519</td>
<td>47</td>
<td>73</td>
<td>186</td>
</tr>
<tr>
<td>Day 1</td>
<td>3.13</td>
<td>0.154</td>
<td>1263</td>
<td>60</td>
<td>192</td>
<td>216</td>
</tr>
<tr>
<td>Day 2</td>
<td>6.51</td>
<td>0.125</td>
<td>1402</td>
<td>82</td>
<td>158</td>
<td>171</td>
</tr>
<tr>
<td>Day 3</td>
<td>7.89</td>
<td>0.060</td>
<td>1041</td>
<td>84</td>
<td>146</td>
<td>144</td>
</tr>
</tbody>
</table>

LDL was isolated using density gradient ultracentrifugation of the patient EDTA plasma for 20 h at 4°C. The plasma was obtained on 4 different days before and during patient supplementation with vitamin E. LDL antioxidant and PUFA content and the lag-phase of conjugated diene accumulation were measured within 24 h of isolating the LDL. Lag-phase duration was measured at LDL concentration of 0.12 mg total cholesterol/ml and Cu\text{2+} concentrations shown. The results shown are means of two experiments.
This analysis points out that α-tocopherol should always function as an antioxidant, if the concentration of co-antioxidants is high enough to efficiently recycle α-tocopheroxy radical back to α-tocopherol. Remarkable efficiency of ascorbate and bilirubin in eliminating α-tocopheroxy radical (20, 21) together with their high concentration in human plasma, implies that, in vivo, in the presence of high concentrations of highly effective co-antioxidants, α-tocopherol should normally behave as an antioxidant independently of the oxidative conditions. Indeed, α-tocopherol typically acts antioxidatively under in vivo conditions (48). On the other hand, no antioxidative action of α-tocopherol has been repeatedly found in vitro in isolated LDL unsupplemented with this antioxidant (7, 8, 11-18). Our scheme of α-tocopherol action explains this contradiction simply by the presence of high concentrations of co-antioxidants in biological fluids in vivo and by their virtual absence in vitro in oxidizing LDL samples (except ubiquinol-10 which is quantitatively not the major co-antioxidant for α-tocopherol (21, 46)).

We postulate that the antioxidant activity of α-tocopherol is its major activity in human plasma and plasma lipoproteins in vivo. It may, however, evolve into prooxidant, when the co-antioxidants are exhausted under mild oxidative conditions. Such a transformation might occur in vivo at chronic oxidative stress induced, for...
example, by iron overload in thalassemia (49) or by copper overload in Wilson's disease (50). It appears that plasma lipoproteins can be sequestered from water-soluble antioxidants in the extracellular fluid within the arterial wall (2). It is therefore likely that such a switching between anti- and prooxidant activities of α-tocopherol might occur in the arterial wall rather than in whole plasma. It remains to be shown whether this process is physiologically or pathologically relevant.

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