Oxidation of Low Density Lipoproteins Leads to Disturbance of Their Binding with α-Tocopherol

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Abstract. The dynamics of binding of exogenous α-tocopherol (α-T) added to native or oxidatively modified LDLs (LDLs or oxLDLs) were investigated Venous blood from 31 clinically healthy blood donors (15 males and 16 females) was used LDLs were isolated by density gradient ultracentrifugation LDLs were oxidized *in vitro* by $CuSO_4$ LDLs or oxLDLs were enriched with exogenous α-T (initial concentrations 0, 10, 20 50, or 100 nmol per mg protein) The contents of α-T in LDLs or in oxLDLs were measured by HPLC Lag-phase of LDL oxidation before or after saturation with α-T was recorded Correlation analysis of the lag-phase of LDL oxidation and α-T content in LDLs was carried out by the method of Esterbauer et al The experimental results demonstrated that (i) α-T was incorporated into native LDLs to a higher extent as compared to oxLDLs (ii) A saturation of LDLs and oxLDLs with α-T was observed (iii) A positive correlation was observed between the duration of the lag-phase of LDL oxidation *in vitro* and the content of α-T in LDLs (iv) Based on LDL saturation with α-T the persons could be classified in two groups LDLs from group I of 26 persons were found to incorporate exogenous α-T to the extent of 1 8 to 3 times its initial concentration, LDLs from group II of 5 persons incorporated little or no exogenous α-T In the first group, oxidation of LDLs lead to a considerable decrease in α-T-dependent variable k and to a moderate reduction of α-T-independent variable a in the equation of Esterbauer et al *lag-phase* = k [*α-tocopherol*] + a In the second group, oxidation of LDLs lead to insignificant changes in k, as well as in a (v) According to the levels of k and a the native LDLs from the second group of 5 persons were very close to oxLDLs from the first group of 26 persons Presumably, native LDLs from the

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second group of persons were initially oxidatively modified, and probably this will be a risk group in relation to atherogenic disorders

Key words: Low density lipoproteins – Oxidative modification – α-Tocopherol

Introduction

Increasing evidence has indicated that free radical reactions may be involved in oxidative modifications of low density lipoproteins (LDL) (Steinbrecher et al 1984, Steinberg et al 1989, Witztum and Steinberg 1991 Esterbauer et al 1992a, Packer 1992, Regnstrom et al 1992 Lyons 1993, Witztum 1994) Oxidatively modified LDLs (oxLDLs) may promote atherosclerosis by different mechanisms by scavenger cell receptor uptake, by cytotoxic effect and following endothelial dysfunction chemotaxis of circulating monocytes and inhibition of their migration (Steinbrecher et al 1984 Steinberg et al 1989 Witztum and Steinberg 1991, Esterbauer et al 1992a,b Packer 1992, Regnstrom et al 1992, Lyons 1993, Campbell and Campbell 1994, Van Berkel et al 1994, Witztum 1994) A number of recent studies have shown the protective effect of antioxidant vitamins on the oxidative modifications of LDLs (Esterbauer et al 1989a,b, 1992a,b, Cornwell et al 1990 Harats et al 1990, Dieber-Rotheneder et al 1991 Packer 1992 Ingold et al 1993 Parthasarathy and Santanam 1994) Several mechanisms for antioxidant defence of LDLs have been discussed

consumption of the antioxidants during their interaction with free radicals and activated oxygen species (Halliwell and Gatteridge 1984, Niki 1987, Sies 1991),

elimination of the lipophilic antioxidants by complex formation with free fatty acids or with lysophospholipids generated as products of the oxidative modifications of LDLs (Erin et al 1984, Urano et al 1987),

– elimination of the lipophilic antioxidants by complex formation with cholesterol esters (Urano and Matsuo 1987, Kagan 1990),

– disturbance of interactions between the reducing plasma equivalents and lipophilic LDL antioxidants this would prevent the recycling of lipophilic LDL antioxidants (Kagan et al 1990a 1992 Hiramatsu and Packer 1991, Packer 1992, Witztum 1994),

– disturbance of the possibilities of binding of lipophilic antioxidants with LDLs (Lin 1993, Kaygen and Traber 1993)

The present work focused on the latter mechanism, because a disturbance of the binding of lipophilic antioxidants with LDLs may influence all the other possible mechanisms of LDL antioxidative defence We studied the dynamics of binding of exogenous α-tocopherol added to native (nonmodified) and to oxidatively modified LDLs

Materials and Methods

Chemicals

All reagents, analytical grade, were obtained from Aldrich Chem. Co., Henkel Co., Merck, and Sigma Chemical Company.

Study Design

Thirty-one clinically healthy blood donors (15 males and 16 females, mean age 34 years, range 24-39) without hypertension, coronary artery disease, hypercholesterolaemia, hyperlipidaemia, hypertriglyceridaemia, diabetes, liver or kidney diseases were included in present study. Blood was obtained from the cubital vein after overnight fast.

Isolation of LDLs

LDLs were isolated by sequential ultracentrifugation in a Beckman L8 55 ultracentrifuge. Briefly, venous blood was taken from each person, after overnight fast, into Vacutainer tubes containing K-EDTA (1 mg/ml blood, final concentration). The plasma was collected after centrifugation and was dialyzed against PBS (10 mmol/l, pH 7.4, 4 °C, for 6 h). LDL fraction was isolated by ultracentrifugation in a density gradient (KBr). The following solvent density interval was used: $d = 1.019 - 1.063$ g/ml, to purify LDLs. The isolated LDL fraction was dialysed extensively against PBS (10 mmol/l, pH 7.4, 4 °C, for 6 h with 2 changes of the buffer). The purity of LDL fraction was checked by electrophoresis. The isolated LDLs gave a single band on 1% agarose gel electrophoresis, and contained only intact apoprotein B. LDLs were used in experiments immediately after isolation. For experiments, LDLs were resuspended in PBS (10 mmol/l, pH 7.4).

HPLC analysis of α-tocopherol content

α-tocopherol was extracted from LDL fraction as described by Lang et al. (1986) and was assayed by HPLC using a C-18 column (25 × 4.1 mm). The eluent was methanol-ethanol 1 : 9 (v/v), 20 mmol/l lithium perchlorate. The flow rate was 1 ml/min and the injected volume was 20 μl. The eluate was monitored by spectrofluorimetric detector – $\lambda_{ex} = 292$ nm, $\lambda_{em} = 325$ nm.

Oxidation of blood plasma

Blood plasma was oxidized according to the method described by Esterbauer et al. 1989b. In brief, the blood plasma was adjusted to pH 7.4 with 50 mmol/l K, Na-phosphate buffer. $CuSO_4$ (10 μmol/l) was added to the plasma followed by incubation for 18 h at 37 °C. After the incubation, the oxidized LDLs (oxLDLs) were isolated from the plasma.

LDLs isolated from nonoxidized blood plasma were used as control. To test whether oxidation of LDLs occurred during the incubation of the plasma with

$CuSO_4$, the electrophoretic mobility of LDL particles in agarose gel was measured The design of the experiment is shown in Diagram 1

Blood plasma

⇓ ⇓

Control aliqouts	Oxidation of blood plasma with $CuSO_4$

⇓ ⇓

Isolation of LDLs Isolation of oxLDLs(LDLs)
(by ultracentrifugation)

- Control of degree of LDL oxidation – by determination of electrophoretic mobility of LDL particles in agarose gel before and after addition of $CuSO_4$

⇓ ⇓

Addition of exogeneous α-tocopherol to LDLs or ox LDLs suspensions

0 10 20 50 100

nmol α-tocopherol/mg LDL protein

Incubation in water-bath – at 37°C for 60 min

⇓ ⇓

Isolation of LDLs or oxLDLs from each suspension by ultracentrifugation

⇓ ⇓

- Determination of α tocopherol bound to LDLs or to oxLDLs
- Recording of lag-phase of LDL(oxLDL) oxidation by $CuSO_4$

Diagram 1. Experimental procedure for the isolation of native LDLs and oxidatively modified LDLs from blood plasma

Native LDLs and oxLDLs were used in further experiments

Enrichment of native and oxidized LDLs with exogenous α-tocopherol

α-Tocopherol was added to 2 ml of LDL suspension (1 mg LDL protein/ml), followed by incubation for 120 min at 37°C, in a nitrogen atmosphere α-Tocopherol was dissolved in ethanol (5 μl ethanol/2 ml LDL suspension)

Thereafter, the LDL suspension was centrifuged as described above to isolate LDL fraction (Diagram 1) LDLs were resuspended in PBS to a final concentration of protein of 0.5 mg/ml, and the amount of α-tocopherol was analyzed by HPLC (Lang et al. 1986).

Oxidation of native and oxidized LDLs by $CuSO_4$

LDLs (25 μg protein/ml) were oxidized by 1.67 μmol/l $CuSO_4$ and continuously monitored sprectrophotometrically at 234 nm at 30 °C to follow the formation of conjugated diens (Esterbauer et al. 1989b). The lag-phase of the reaction was calculated.

Protein concentration was measured by the method of Lowry et al. (1951) with bovine serum albumine as a protein standard.

Statistical analysis

The results were expressed as mean ± S.D. The data were analyzed by ANOVA with statistical significance of differences between the experimental groups determined by Dunnett's test. Statistical significance was assessed at $P < 0.05$.

Results

Binding of exogenous α-tocopherol with native and oxidized LDLs

On the basis of the degree of LDL and oxLDL saturation with α-tocopherol, the blood donors could be classified into two groups: "α-tocopherol-binders" (group I) and "α-tocopherol-nonbinders" (group II). The differences in the degree of LDL or oxLDL saturation with exogenous α-tocopherol between group I and II were statistically significant ($p < 0.01$).

The results of LDL or oxLDL saturation by α-tocopherol in group I of 26 persons (11 males and 15 females) are shown in Fig. 1*A*. Addition of α-tocopherol in concentrations between 10 and 100 nmol/mg LDL protein resulted in the saturation of LDLs and oxLDLs with this antioxidant. Native LDLs were saturated after addition of 50 (or more) nmol exogenous α tocopherol per mg protein (Fig. 1*A*). OxLDLs (isolated after oxidation of blood plasma by Cu^{2+} *in vitro* – see Diagram 1) were saturated with 20 (or more) nmol exogenous α-tocopherol per mg protein (Fig. 1*A*). Also, native LDLs bound exogenous α-tocopherol 1.8 to 3 times its initial steady-state concentration in the lipoproteins (15.1±6.3 nmol α-tocopherol/mg protein). However, oxLDLs bound exogenous α-tocopherol 1.4 to 2.3 times (maximum) its initial concentration in oxLDL (9.0±4.5 nmol α-tocopherol/mg protein). Maximum concentrations of α-tocopherol measured in LDLs or oxLDLs (after addition of 100 nmol exogenous α-tocopherol per mg LDL-protein) were 43.5 ± 12.7 nmol/mg protein or 22.5 ± 7.5 nmol/mg protein, respectively.

The results of LDL or oxLDL saturation by α-tocopherol in group II of 5 persons (4 males and 1 female) are presented in Fig. 1*B*. In this case, native LDLs were saturated at 20 (or more) nmol exogenous α-tocopherol per mg protein. Native

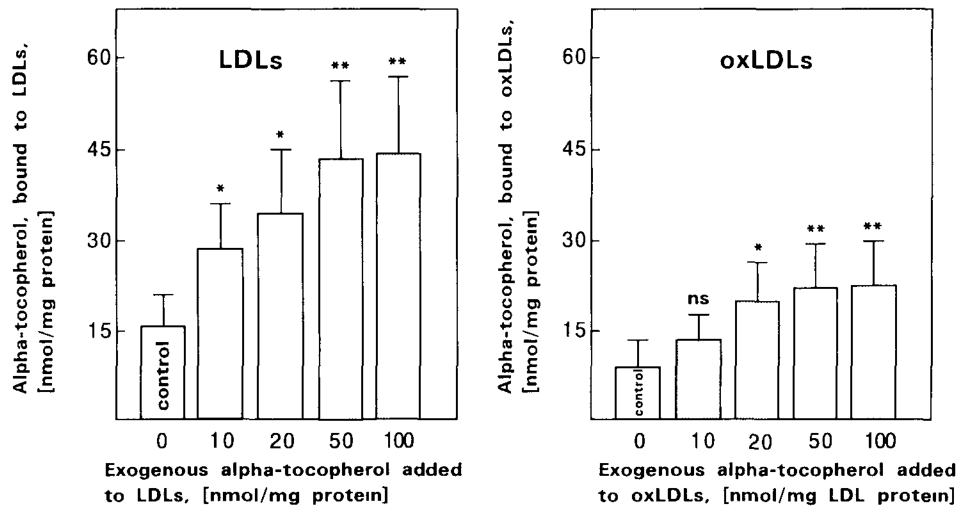

Figure 1A. Dependence of the degree of binding of α-tocopherol to native LDLs or oxLDLs on exogenously added α-tocopherol in group I of clinically healthy blood donors 'α-tocopherol-binders' (* $p < 0.05$ ** $p < 0.01$ *vs* control *ns* no significance *vs* control)

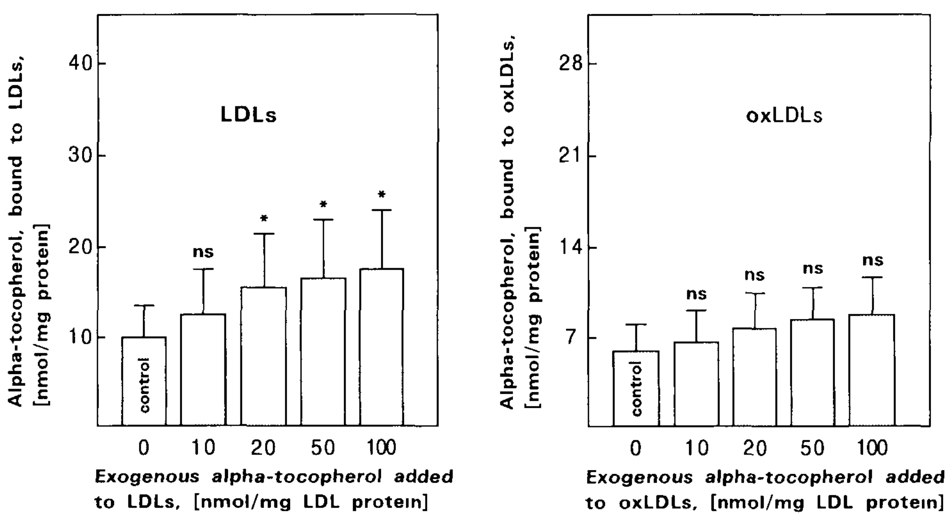

Figure 1B. Dependence of the degree of binding of α-tocopherol to native LDLs or oxLDLs on exogenously added α-tocopherol in group II of clinically healthy blood donors – "α-tocopherol-nonbinders" (* $p < 0.05$ *vs* control *ns* – no significance *vs* control)

LDLs from group II of clinically healthy blood donors were found to incorporate exogenous α-tocopherol only to the extent of 1.3–1.7 times its initial concentration in lipoproteins (10.0 ± 3.5 nmol α-tocopherol/mg protein). oxLDLs from this group of persons incorporated practically no exogenous α-tocopherol. In this case maximum concentrations of α-tocopherol measured in LDLs or oxLDLs (after addition of 100 nmol exogenous α-tocopherol per mg LDL-protein) were 17.0 ± 7.1 nmol/mg protein or 8.7 ± 2.8 nmol/mg protein, respectively.

A comparison of the results shown in Figs. 1A and 1B suggests that:

- in both groups ("α-tocopherol-binders" and "α-tocopherol-nonbinders") exogenous α-tocopherol was incorporated into native LDLs to a higher extent as compared to oxLDLs;
- native LDLs from group I (26 "α-tocopherol binders") incorporated exogenous α-tocopherol about twice as much as native LDls from group II (5 "α-tocopherol nonbinders");
- the degree of α-tocopherol saturation of native LDLs from group II is similar to the degree of α-tocopherol saturation of oxLDLs from group I.

Correlation between the lag phase of LDL oxidation by $CuSO_4$ and the content of α-tocopherol in LDLs or oxLDLs after saturation

A significantly decreased susceptibility of LDLs to oxidation after saturation with α-tocopherol ($p < 0.01$) was demonstrated by changes in the kinetics of conjugated diene formation following incubation of LDLs or oxLDLs with $CuSO_4$. The changes were characterized by an increased lag-phase of LDL oxidation after addition of α-tocopherol (Figs. 2A, B). It was shown that in group I (Fig. 2A) lag phase of LDL oxidation by $CuSO_4$ increased significantly after addition of increased concentrations of exogenous α-tocopherol to native LDLs ($p < 0.01$). Lag-phases of lipoprotein oxidation by $CuSO_4$ was increased minimally ($p > 0.05$) after addition of increased concentrations of exogenous α-tocopherol to oxLDLs from group I, and to native LDLs and oxLDLs from group II (Fig. 2B).

A highly positive correlation was shown between the duration of the lag phase of LDL oxidation by induced $CuSO_4$ and the content of α-tocopherol bound to LDLs or oxLDLs (Fig. 3). The mathematical modeling of the experimental results presented in Figs. 1 and 2 was carried out by the method of Esterbauer et al. 1992a. The equation describing the relationship between α-tocopherol concentration and the lag-phase of LDL oxidation was expressed as *lag-phase* = $k \cdot$ [*α-tocopherol*] + *a*, where k was the α-tocopherol-dependent constant (efficacy constant of α-tocopherol) and was calculated from the slope of the correlation line; a was the α-tocopherol independent variable in minutes calculated from the intercept of the correlation line with y axis, and [α-tocopherol] was the concentration of α-tocopherol added to LDLs. The same methodological approach was employed in our study to calculate the above mentioned parameters k and a for native LDLs and oxLDLs.

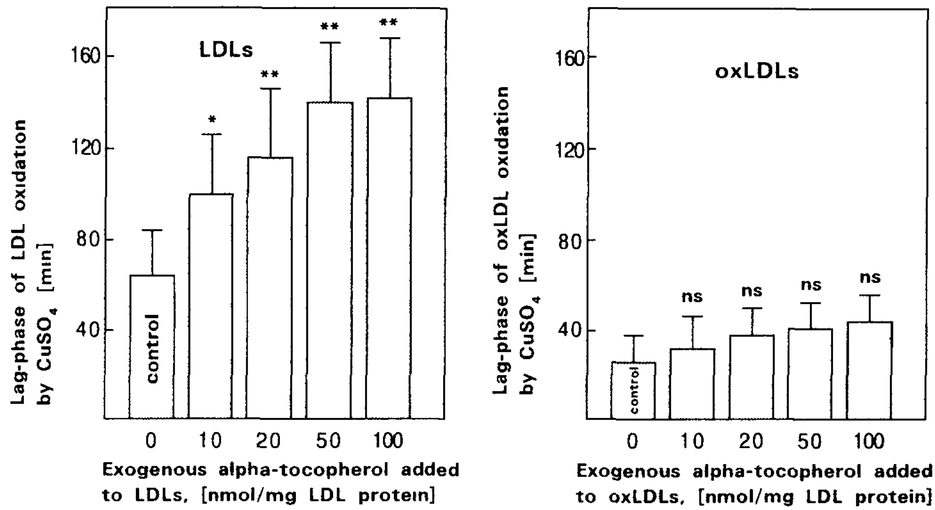

Figure 2A. Dependence of the lag-phase of LDL or oxLDL oxidation by $CuSO_4$ *in vitro* on the degree of binding of α-tocopherol with respective lipoproteins in group I of clinically healthy blood donors – "α-tocopherol-binders" (* $p < 0.05$ *vs* control, *ns* - no significance *vs* control)

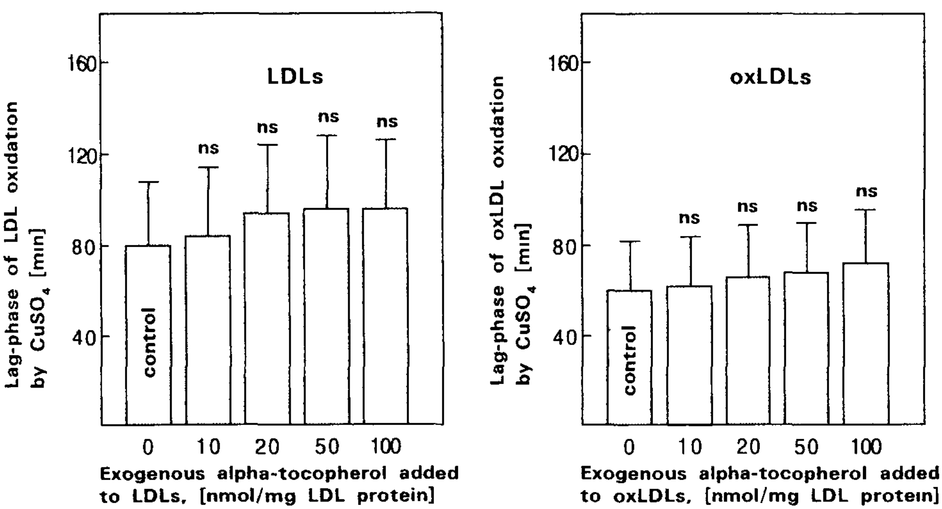

Figure 2B. Dependence of the lag-phase of LDL or oxLDL oxidation by $CuSO_4$ *in vitro* on the degree of binding of α-tocopherol with respective lipoproteins in group II of clinically healthy blood donors – "α-tocopherol-nonbinders" (*ns* – no significance *vs* control)

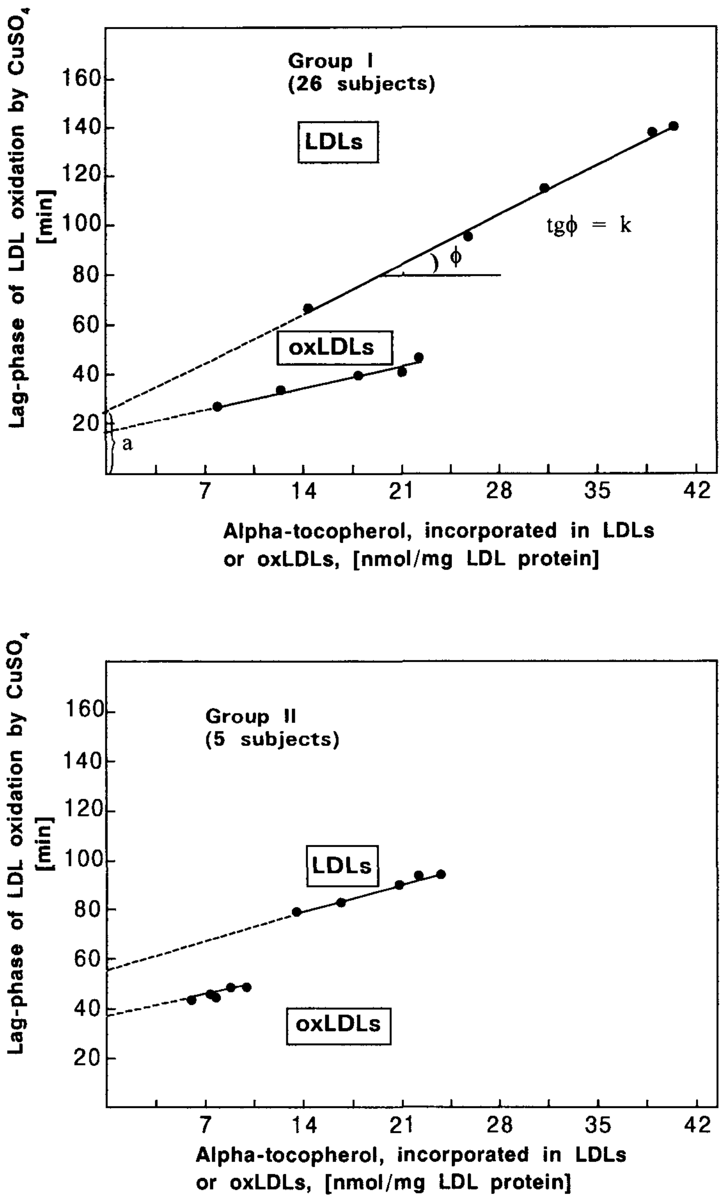

Figure 3. Linear correlation between α-tocopherol content in LDLs or oxLDLs and lag-phase of lipoprotein oxidation by $CuSO_4$. *A* – First group of clinically healthy blood donors. *B* – Second group of clinically healthy blood donors.

In the first group (α-tocopherol-binders) k values were 2 13 $\pm$ 0 58 and 0 74 $\pm$ 0 22 for LDLs and oxLDLs respectively and the a values were 22 6 $\pm$ 8 1 for LDLs and 19 0 $\pm$ 7 2 for oxLDLs In the second group (α tocopherol-nonbinders) k values were 1 18 $\pm$ 0 30 for LDLs and 1 06 $\pm$ 0 37 for oxLDLs and the a values were 51 6 $\pm$ 15 5 for LDLs and 37 4 $\pm$ 11 2 for oxLDLs

Discussion

Our experiments showed that native LDLs were able to bind much more exogenous α tocopherol as compared to oxLDLs A saturation of native LDLs and oxLDLs with α tocopherol was observed In the case of multi or monolammelar liposomes (prepared from different lipids) no saturation with α-tocopherol was observed in the range of α tocopherol concentrations used (Kagan et al 1990b c) These results are in good agreement with those reported by Esterbauer et al (1992a b)

The relationship between the lag phase of *in vitro* LDL oxidation by Cu^{2+} and the amount of exogenous α tocopherol added to the blood is described by the linear equation (Esterbauer et al 1992a) *lag phase in minutes* = k [α *tocopherol concentration*] + a (where k and a were characteristic of subject specific constants by which the oxidation resistance of the LDLs was determined)

In our experiments a positive correlation was observed (r = 0 94 $p <$ 0 001) between the duration of lag-phase of LDL oxidation *in vitro* and the content of α-tocopherol in LDLs

According to the degree of exogenous α tocopherol binding to LDLs or oxLDLs the clinically healthy donors may be classified into two groups The first group (α tocopherol binders) expressed a significantly higher capacity of LDLs to bind α-tocopherol In this group the values of α tocopherol dependent variable k for LDLs were considerably higher ($p <$ 0 001) than the k values for oxLDLs The values of α tocopherol-independent variable a were slightly decreased ($p <$ 0 05) after LDLs oxidation LDLs from the second group of subjects (α-tocopherol nonbinders) exhibited a poor affinity for exogenous α-tocopherol In this group the values of α tocopherol dependent variable k as well as the values of α tocopherol independent variable a for oxLDLs were not significantly different from those of native LDLs

At present however it is uncertain why constants for an individuals are different and whether they change with age lifestyle and dietary habits It is intriguing to speculate that if a is low the α-tocopherol intake may lead to an increase of antioxidant defence of LDLs (estimated by the lag phase time) For such subjects α-tocopherol may function as the major LDL protector during *in vitro* oxidation On the other hand for subjects with low levels of k, even megadoses of α-tocopherol may bring only minimum protective effect against LDL oxidation (Esterbauer et al 1989b 1992a)

The amount of polyunsaturated fatty acids (PUFA s) the ratio of PUFAs to saturated fatty acids, cholesterol content, mobility of α-tocopherol, and structure of apo-B are factors which may influence the values of k and a (Massay 1984, Cornwell et al 1990)

All these findings may suggest that if interaction of α-tocopherol with LDLs is a nonspecific process (determined only by the incorporation of antioxidant in the lipid phase of LDLs), supplementation of LDLs with α-tocopherol should result in almost the same observations in different biological subjects, as well as in native and oxidatively modified LDLs This however was not the case in our experiments Probably, α-tocopherol was bound to LDLs not only by incorporation into the lipoprotein lipid phase, but also by "specific protein"-mediated interactions

As the results for *in vitro* oxidized LDLs are very similar to *in vivo* oxidatively modified LDLs in atherogenesis, factors determining the levels of k and a cast doubt on the necessity and efficiency of α-tocopherol in the prophylaxis and treatment of atherogenic disorders This assumption will further be tested in our future experiments

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