RRR-α-tocopherol can be substituted for by Trolox in determination of kinetic parameters of LDL oxidizability by copper[1]

Andreas R. Bergmann, Pilar Ramos,[2] Hermann Esterbauer,[3] and Brigitte M. Winklhofer-Roob[4]

Institute of Biochemistry, Karl-Franzens University of Graz, Schubertstrasse 1, A-8010 Graz, Austria

Abstract LDL resistance to oxidation is characterized by the lag time that precedes the rapid generation of conjugated dienes in copper-induced oxidation. Lag time (y) is described by the equation $y = kx + a$, where k is the efficacy constant of α-tocopherol (α-TOH), x the α-TOH content of LDL, and a the α-TOH-independent variable, all of which show considerable between-subject variability. To answer the question of whether loading of LDL with Trolox can substitute for the more time-consuming loading with RRR-α-TOH in large scale studies focusing on determination of k and a values, LDL from 15 healthy subjects was loaded in vitro with 0–50 mol Trolox/mol LDL or 0–250 mM RRR-α-TOH. In addition, five of these subjects were supplemented in vivo with 800–1200 IU/d RRR-α-TOH for 17 days and k and a were determined. The ratios k_{Trolox}:$k_{\alpha\text{-TOH}}$ and a_{Trolox}:$a_{\alpha\text{-TOH}}$ were calculated. The k_{Trolox} was only slightly higher than $k_{\alpha\text{-TOH}}$, in contrast to α_{Trolox}, which was almost twice as high as $a_{\alpha\text{-TOH}}$, indicating that, in the case of Trolox, a includes the contribution of α-TOH to the lag time. In the case of α-TOH, a includes only the additional antioxidants that occur naturally in LDL. The ratios k_{Trolox}:$k_{\alpha\text{-TOH}}$ and a_{Trolox}:$a_{\alpha\text{-TOH}}$ showed little variability, both between subjects and between in vitro and in vivo experiments. A close correlation was found between $k_{\alpha\text{-TOH}}$ and $a_{\alpha\text{-TOH}}$, calculated using this ratio, and $k_{\alpha\text{-TOH}}$ and $a_{\alpha\text{-TOH}}$, obtained in our experiments. We conclude that from this ratio, $k_{\alpha\text{-TOH}}$ and $a_{\alpha\text{-TOH}}$ can be reliably calculated when k_{Trolox} and a_{Trolox} are determined experimentally, thus allowing us to obtain k and a values in a more convenient way.—**Bergmann, A. R., P. Ramos, H. Esterbauer, and B. M. Winklhofer-Roob.** RRR-α-tocopherol can be substituted for by Trolox in determination of kinetic parameters of LDL oxidizability by copper. *J. Lipid Res.* 1997. **38:** 2580–2588.

Supplementary key words low density lipoprotein • α-tocopherol • Trolox • oxidation • lag time • copper

Resistance of isolated LDL in vitro to a defined oxidative stress that is generated by Cu(II) ions is frequently determined in a well-described model system (1). LDL resistance to oxidation is characterized by *i*) the lag time that precedes the rapid generation of conjugated dienes (CD), *ii*) the maximum rate of oxidation, and *iii*) the maximum formation of conjugated dienes. That LDL resistance to oxidation in this model shows substantial between-subject variability in healthy individuals has been reported in a growing number of studies (2–8). Major contributing factors are the LDL content of endogenous antioxidants such as α-tocopherol (2–5), ubiquinol (9), and carotenoids (5, 6), along with the fatty acid content of LDL (7, 8), apolipoprotein B (10) and the presence of dense LDL subfractions (11).

The most frequently used index of resistance to oxidation of LDL is the lag time. Even though there is a linear relation between lag time and α-tocopherol content of LDL in subjects supplemented with vitamin E (12), the α-tocopherol content of LDL does not, by itself, predict the length of the lag time of a given LDL sample (12). Lag time (y) has been described by the equation $y = kx + a$ where k is the efficacy constant of α-tocopherol, x the α-tocopherol content of LDL, and a the α-tocopherol-independent variable (12, 13). In line with the above, the α-tocopherol-independent variable, for instance, may comprise different antioxidants present in LDL other than α-tocopherol. Also, the efficacy of 1 mol α-tocopherol in protecting a single LDL particle is likely to be influenced, for instance, by the PUFA content of LDL, perhaps as a direct function of the number of bisallylic double bonds. However, it has been observed that both k and a show extreme between-subject variability (13), indicating that more

Abbreviations: α-TOH, α-tocopherol; CV, coefficient of variation; CD, conjugated dienes.

[1] Presented in part at the SFRR Europe Summer Meeting, 1997, in Abano Terme, Italy.

[2] Present address: Department of Biomedical Sciences, Faculty of Experimental and Technical Sciences, University San Pablo, CEU, Madrid, Spain.

[3] Deceased January 7, 1997, before completion of the manuscript.

[4] To whom correspondence should be addressed.

variables may be involved, as recently reviewed (12). It has been suggested that both k and a are indeed characteristic subject–specific constants that determine the individual LDL resistance to in vitro oxidation, but an individual's constants may change with dietary habits and age (12). Clearly, further work is needed and large groups of subjects should be studied repeatedly.

So far, in studies that focused on the effect of α-tocopherol on LDL resistance to oxidation, subjects have been supplemented in vivo (2–4), or plasma has been incubated with α-tocopherol in vitro (5, 14). On the other hand, the vitamin E analog Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) (15) has been used in different in vitro (16–20) and in vivo studies (20–22), because of its water-solubility due to the absence of the phytyl side chain, which constitutes an advantage over α-tocopherol.

We proposed that this advantage can also be applied for loading of LDL samples with vitamin E in vitro. The aim of this study was, therefore, to answer the question of whether loading of LDL with Trolox can substitute for the more time-consuming in vitro loading with α-tocopherol for large-scale studies, focusing on determination of k and a values.

METHODS

Subjects

Fifteen clinically healthy subjects, seven males and eight females, aged 22 to 34 years, living in the area of Graz, Austria, donated plasma which was used for the experiments that focused on in vitro loading of LDL with Trolox and RRR-α-tocopherol. All subjects were on a usual diet and did not take vitamin supplements. Five of these subjects, two males and three females, aged 22 to 34 years, volunteered also for oral supplementation with RRR-α-tocopherol (subjects #11–15). The study was conducted according to the Declaration of Helsinki and informed consent was obtained from the subjects who volunteered for this study.

Reagents

All reagents used were of analytical grade or better, and were purchased from Merck (Darmstadt, Germany) or Sigma Chemicals (St. Louis, MO). All solutions were prepared using ion-exchanged laboratory water filtered through a Millipore Norganic cartridge (Millipore Corporation, Bedford, MA).

Preparation of plasma and LDL

Blood was drawn after an overnight fast and collected into plastic tubes containing an aqueous solution of 10% EDTA to give a final concentration of 0.1% EDTA. Plasma was obtained by three consecutive steps of centrifugation: 1.800 g for 10 min, 5.000 g for 5 min, 13.000 g for 10 min, at 4°C. If the plasma was not immediately processed, a 60% sucrose solution was added to give a final concentration of 0.6%, and the samples were kept at −80°C (23). Plasma treated like this can be processed within 2 weeks without a detectable loss of antioxidants and changes in lag time (24). LDL was prepared according to Gieseg and Esterbauer (25) with a minor modification, i.e., the plasma solution was prepared by dissolving 1.5264 g of solid potassium bromide in 4 ml of defrosted EDTA-plasma. LDL was prepared by a single-step discontinuous gradient ultracentrifugation in a Beckman NVT65 rotor at 60.000 rpm for 2 h at 10°C (23). LDL was finally filtered through a 0.45 μm sterile filter (Corning, Japan) into an evacuated glass vial (Techne Vial, Mallinckrodt-Diagnostica, Petten, Holland) and stored at 4°C under argon in the dark for a maximum of 2 weeks (24).

Determination of antioxidants in plasma and LDL and lipid composition of LDL

Vitamin E and carotenoid content of plasma and LDL were determined by HPLC, as described recently (26). The antioxidant content of LDL was expressed as mol antioxidant/mol LDL and that of plasma as μmol/L plasma, respectively. The lipid composition of LDL was determined as described (26).

LDL oxidation

A volume of 0.5 mL LDL was desalted and made EDTA-free by gel-filtration, using Econo-Pac 10DG columns (Bio-Rad, Richmond, CA). The columns were equilibrated with 20 mL PBS (10 mM sodium phosphate buffer, pH 7.4, containing 0.15 M sodium chloride). The same PBS served as eluting buffer. Then the LDL concentration in the PBS solution was estimated by determination of total cholesterol with a CHOD-PAP enzymatic test kit (Boehringer-Mannheim, Mannheim, Germany). The LDL concentration was expressed as μM, assuming an LDL molecular weight of 2.5×10^6 Da and a cholesterol content of 31.6% by weight. For the oxidation experiments, LDL was used at a concentration of 0.1 μM in PBS equal to 0.25 mg LDL total mass/mL. The oxidation process was induced with an aqueous solution of 100 mM $CuSO_4$. This solution was prepared fresh for each experiment and added to the quartz cuvettes to give a final concentration of 1.67 μM. Reagents were pipetted into the cuvettes in the sequence PBS–LDL–Trolox (when used)–$CuSO_4$. Oxidation was monitored by recording the CD absorbance at 234 nm (1) in Beckman DU-640 spectrophotometer, equipped with an auto-cell holder. CD absorbance

curves for six samples could be measured simultaneously. Reaction temperature was kept at 37°C and the absorption was measured every 2 to 5 min. From the CD absorbance curve, lag time, maximum rate of oxidation and maximum CD absorbance were determined as the indexes of LDL oxidation, as described (27).

In vitro loading with α-tocopherol

To increase the vitamin E content of LDL in vitro, plasma of 15 different subjects was loaded with RRR-α-tocopherol. For this purpose, RRR-α-tocopherol was dissolved in ethanol to give solutions of 50, 75, 150, and 250 mM, which were covered with argon gas and kept in the dark at 4°C for no longer than 2 weeks. For the loading experiments, 4 mL of plasma from each subject was incubated with 20 μL of the α-tocopherol solution in pyrex tubes in a slow-shaking water-bath at 37°C for 3 h, according to Esterbauer et al. (14). A blank with plasma and ethanol alone was simultaneously prepared in the same way. Where necessary, the ratio plasma:ethanol was reduced to 3 ml:15 μL. In each individual LDL sample, the concentration of α-tocopherol reached in LDL during loading was determined.

In vitro loading with Trolox

Trolox was obtained from Aldrich Chemical Company Inc., Milwaukee, WI. A stock solution of 0.1 mM was prepared, covered with argon gas, and stored in the dark at 4°C for a maximum of 2 weeks. Immediately before the addition of $CuSO_4$, Trolox was added to LDL of the 15 different donors to give a final concentration of 0, 10, 30, and 50 mol Trolox/mol LDL, respectively, in the sequence described above.

In vivo supplementation with α-tocopherol

Blood was drawn after an overnight fast at study entry, and 3, 6, 10, and 17 days after oral supplementation with RRR-α-tocopherol. Two of the five subjects enrolled took 800 I.U. per day (2 softgels) and three 1200 I.U. per day (3 softgels) RRR-α-tocopherol for 17 days (Etocovit, Richter Pharma, Wels, Austria). These supplements were taken with meals and the subjects were asked not to change their usual dietary habits during the study. In addition, antioxidant content and resistance to oxidation of LDL obtained at day 0, 3, 6, 10, and 17 of in vivo supplementation were determined.

Determination of *k* and *a* values

From the equation $y = kx + a$ (12, 13), where y is the lag time, k as the efficacy constant of vitamin E and a as the vitamin E-independent variable were determined for α-tocopherol and, similarly, for Trolox. Ratios of k values for Trolox and α-tocopherol (k_{Trolox}:$k_{α\text{-}TOH}$) and of a values (a_{Trolox}:$a_{α\text{-}TOH}$) were calculated for both in vitro and in vivo experiments.

Statistical analysis

Comparisons were made using one-way ANOVA, repeated measures ANOVA, and Student's t-tests, respectively. SigmaStat, version 2.0 (Jandel Scientific, San Rafael, CA), was used for all statistical procedures. P values <0.05 were considered significant. Values are expressed as mean ± SD.

RESULTS

Reproducibility

In order to test the reproducibility of LDL oxidation in the presence of 0–50 mol Trolox/mol LDL, experiments were performed with four different LDL samples, isolated from a plasma pool used in our laboratory and oxidized under conditions described above. Results are shown in **Table 1**. With increasing Trolox concentrations, lag times increased from 77.0 ± 2.94 min to 416.0 ± 10.68 min. The coefficient of variation (CV)

TABLE 1. Lag times obtained in four individual experiments for increasing Trolox concentrations along with parameters of the corresponding regression equation, lag time = $k_{Trolox} + a_{Trolox}$

	Trolox (mol/mol LDL)						
Experiment[a]	0	10	30	50	r^2	k	a
	lag time (min)						
1	77	148	290	415	0.99	6.78	79.88
2	78	152	308	430	0.99	7.11	82.14
3	73	146	296	415	0.99	6.89	77.44
4	80	142	296	404	0.99	6.61	81.70
Mean ± SD	77.0 ± 2.94	147.0 ± 4.16	297.5 ± 7.55	416.0 ± 10.68	0.99 ± 0.002	6.85 ± 0.21	80.3 ± 2.10
CV (%)	3.8	2.8	2.5	2.6	0.2	3.1	2.6

[a]Four different LDL samples were isolated independently from one plasma pool and oxidized with 1.67 μM $CuSO_4$ at 37°C.

TABLE 2. Indexes of resistance to oxidation of LDL loaded in vitro with different Trolox concentrations in 15 independent experiments

		Maximum Rate of Oxidation		Maximum CD Absorbance	
Trolox	Lag Time	ΔA/min	At Time	A234 nm	At Time
mol/mol LDL	*min*		*min*		*min*
0	100 ± 24	0.0131 ± 0.0022	133 ± 34	0.732 ± 0.050	181 ± 44
10	178 ± 26	0.0131 ± 0.0014	206 ± 34	0.721 ± 0.044	257 ± 31
30	338 ± 49	0.0127 ± 0.0020	368 ± 64	0.715 ± 0.051	419 ± 54
50	438 ± 65	0.0124 ± 0.0018	476 ± 67	0.717 ± 0.051	529 ± 67
P value[a]	<0.001	n.s.	<0.001	0.01	<0.001

Results are given as mean ± SD of 15 individual experiments; n.s., not significant. Note that LDL α-tocopherol concentrations were 5.74 ± 0.62 mol/mol.

[a]One-way ANOVA.

of lag times was 2.5%–3.8%. In the regression equation k as the Trolox-dependent and a as the Trolox-independent variables were 6.85 ± 0.21 (CV 3.1%) and 80.29 ± 2.10 (CV 2.6%), respectively, and the average regression coefficient (r^2) was 0.99.

Indexes of resistance to oxidation of LDL

In 15 independent experiments, LDL was loaded in vitro with 0–50 mol Trolox/mol LDL, and plasma was incubated with 0–250 mM RRR-α-tocopherol. Indexes of resistance to oxidation of LDL loaded in vitro with Trolox are shown in **Table 2,** and typical CD absorbance curves are presented in **Fig. 1.** There was a significant increase ($P < 0.001$) in lag time (100 ± 24 min vs. 438 ± 65 min), time of maximum rate of oxidation (133 ± 34 min vs. 476 ± 67 min), and time of maximum CD absorbance (181 ± 44 min vs. 529 ± 67 min). The maximum rate of oxidation (propagation rate) did not change significantly, whereas the maximum CD absorbance showed a significant decrease from 0.732 ± 0.050 to 0.717 ± 0.051 ($P = 0.01$). The corresponding results for in vitro α-tocopherol loading are shown in **Table 3** (upper panel). In contrast to the significant increases ($P < 0.001$) in lag time, time of maximum rate of oxidation, and time of maximum CD absorbance, with increasing α-tocopherol concentrations the maximum rate of oxidation decreased significantly from 0.0137 ± 0.0029 to 0.0116 ± 0.0026 ($P < 0.001$), whereas the maximum CD absorbance did not change. Supplementation of five different donors (subjects #11–15) with 800–1200 IU/d RRR-α-tocopherol for 17 days gave similar results (Table 3, lower panel), compared with the in vitro loading of plasma with RRR-α-tocopherol.

Variables of the regression equation

In **Table 4,** the efficacy constants (k) and the independent variables (a) of Trolox and α-tocopherol for lag times obtained from in vitro loading of plasma from donors #1–10 are presented. Both k and a for Trolox and α-tocopherol, respectively, showed considerable variability, whereas Trolox to α-tocopherol ratios (k_{Trolox}:$k_{\alpha\text{-TOH}}$) and (a_{Trolox}:$a_{\alpha\text{-TOH}}$) turned out to vary to a lesser extent. The efficacy constant k of Trolox was only slightly higher than that of α-tocopherol (k_{Trolox}:$k_{\alpha\text{-TOH}}$, 1.17 ± 0.17), in contrast to the Trolox-independent variable a, which was almost twice as high as the α-tocopherol-independent variable a, as shown by a_{Trolox}:$a_{\alpha\text{-TOH}}$ of 1.89 ± 0.08. **Table 5** shows the efficacy-constants and independent variables for Trolox and α-tocopherol in vitro (k, a) and in vivo (k', a') in subjects #11–15. Also for these subjects, k values for Trolox and α-tocopherol (both in vitro and in vivo) did not differ from each other, whereas a values were almost twice as high for Trolox compared with α-tocopherol.

In **Table 6,** antioxidant combinations in plasma and LDL of five donors (#11–15) and the lipid composition of LDL during supplementation with RRR-α-tocopherol are shown. As expected, α-tocopherol concentrations increased significantly in plasma and LDL ($P < 0.001$).

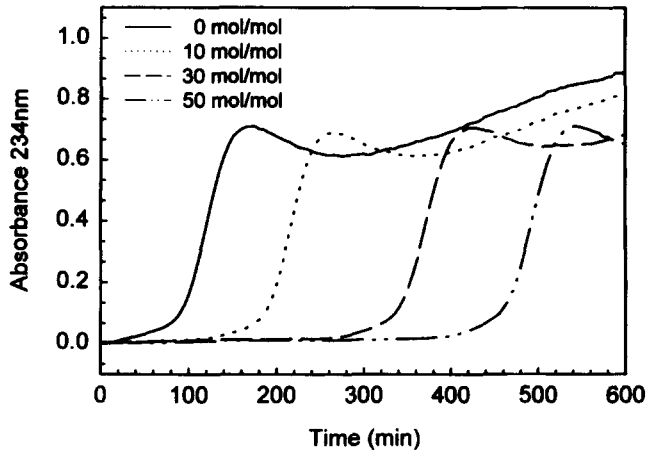

Fig. 1. CD absorbance curves for LDL without and with the addition of 10, 30, and 50 mol Trolox/mol LDL.

TABLE 3. Indexes of resistance to oxidation of LDL loaded in vitro with different α-tocopherol concentrations in 15 independent experiments (upper panel) and of LDL from five donors supplemented with RRR-α-tocopherol for 17 days (lower panel); in addition, the respective α-tocopherol concentrations of LDL are shown

			Maximum Rate of Oxidation		Maximum CD Absorbance	
	LDL α-tocopherol[c]	Lag Time	ΔA/min	At Time	A 234 nm	At Time
	mol/mol	*min*		*min*		*min*
In vitro loaded,[a]						
Concentration[b] (mM)						
0	5.74 ± 0.62	96 ± 19	0.0137 ± 0.0029	123 ± 25	0.753 ± 0.049	169 ± 29
50	10.58 ± 2.76	118 ± 25	0.0132 ± 0.0023	148 ± 28	0.744 ± 0.046	196 ± 33
75	11.55 ± 2.51	126 ± 24	0.0125 ± 0.0018	157 ± 27	0.749 ± 0.051	205 ± 32
150	12.47 ± 2.28	135 ± 26	0.0120 ± 0.0021	167 ± 30	0.748 ± 0.048	216 ± 36
250	15.19 ± 4.37	152 ± 34	0.0116 ± 0.0026	190 ± 40	0.753 ± 0.052	240 ± 48
P value[d]	<0.001	<0.001	<0.001	<0.001	n.s.	<0.001
In vivo supplemented,[e]						
Time (days)[f]						
0	5.74 ± 0.62	98 ± 2	0.0120 ± 0.0019	113 ± 7	0.768 ± 0.034	164 ± 15
3	12.59 ± 1.27	134 ± 24	0.0124 ± 0.0018	146 ± 21	0.798 ± 0.036	199 ± 23
6	14.19 ± 1.97	154 ± 11	0.0110 ± 0.0016	167 ± 17	0.816 ± 0.091	225 ± 15
10	12.99 ± 1.52	151 ± 11	0.0100 ± 0.0012	186 ± 13	0.752 ± 0.064	247 ± 17
17	13.27 ± 1.65	158 ± 6	0.0100 ± 0.0010	199 ± 12	0.782 ± 0.025	261 ± 17
P value[g]	<0.001	<0.001	0.001	<0.001	n.s.	<0.001

[a] Mean ± SD of 15 individual experiments
[b] Concentration of α-tocopherol for loading.
[c] Concentration of α-tocopherol reached in LDL.
[d] One-way ANOVA.
[e] Mean ± SD of 5 subjects.
[f] Days of supplementation with RRR-α-tocopherol (2 subjects took 800, 3 took 1200 IU/d).
[g] Repeated measures ANOVA.

Simultaneously, a significant ($P < 0.001$) decrease in γ-tocopherol concentrations in both plasma and LDL was observed. No significant changes were noted for the other antioxidants. There were no changes in lipid composition of LDL, except a decrease in cholesterol concentrations from day 0 to day 6 ($P < 0.05$), and a decrease in protein concentrations from day 0 to day 10 and day 0 to day 17, respectively ($P < 0.05$). To test the validity of calculating k and a values for α-tocopherol for different donors, the calculated k and a values for α-tocopherol were compared with those obtained experimentally (Fig. 2). For both k and a, close correlations were observed ($r = 0.70$, $P = 0.003$ and $r = 0.97$, $P < 0.0001$, respectively).

TABLE 4. Efficacy constants (k) and independent variables (a) of Trolox and α-tocopherol, respectively, for lag times obtained from in vitro loading of LDL from 10 individual donors

Donor	k_{Trolox}	$k_{\alpha\text{-TOH}}$	k_{Trolox}:$k_{\alpha\text{-TOH}}$	a_{Trolox}	$a_{\alpha\text{-TOH}}$	a_{Trolox}:$a_{\alpha\text{-TOH}}$
1	5.93	5.56	1.07	82.5	42.0	1.97
2	9.03	9.02	1.00	153.5	81.3	1.89
3	5.54	4.81	1.15	78.4	42.0	1.87
4	5.60	3.93	1.43	115.6	61.6	1.88
5	6.04	6.09	0.99	95.6	46.7	2.05
6	8.59	6.24	1.38	116.7	64.5	1.81
7	6.85	4.95	1.38	149.2	80.1	1.86
8	6.91	6.63	1.04	126.3	67.4	1.87
9	6.86	6.59	1.04	121.3	62.9	1.93
10	6.30	5.38	1.17	100.3	56.7	1.77
Mean ± SD	6.77 ± 1.19	5.92 ± 1.38[a]	1.17 ± 0.17	113.9 ± 25.4	60.5 ± 14.1[b]	1.89 ± 0.08
C.V. (%)	17.6	23.3	14.5	22.3	23.3	4.2

To describe the relation between k and a of Trolox and α-tocopherol, respectively, ratios of k_{Trolox}:$k_{\alpha\text{-TOH}}$ and of a_{Trolox}:$a_{\alpha\text{-TOH}}$ were calculated. Note that LDL α-tocopherol concentrations of donors 1–10 were 6.08 ± 0.78 mol/mol (mean ± SD).

[a] Not significantly different vs. k_{Trolox} (Student's t-test).
[b] $P < 0.001$ vs. a_{Trolox} (Student's t-test).

TABLE 5. Values of k and a for Trolox and α-tocopherol in vitro and in vivo

Donor	In Vitro			In Vivo		In Vitro			In Vivo	
	k_{Trolox}	$k_{\alpha\text{-TOH}}$	k_{Trolox}:$k_{\alpha\text{-TOH}}$	$k'_{\alpha\text{-TOH}}$	Trolox:$k'_{\alpha\text{-TOH}}$	a_{Trolox}	$a_{\alpha\text{-TOH}}$	a_{Trolox}:$a_{\alpha\text{-TOH}}$	$a'_{\alpha\text{-TOH}}$	Trolox:$a'_{\alpha\text{-TOH}}$
11	6.35	6.15	1.03	9.07	0.70	97.2	56.8	1.71	51.6	1.88
12	7.25	7.40	0.98	6.77	1.07	110.1	61.7	1.78	62.3	1.77
13	6.97	6.03	1.16	6.63	1.05	106.5	51.5	2.07	59.4	1.79
14	7.23	5.30	1.36	6.62	1.09	91.7	47.3	1.94	49.1	1.87
15	7.17	5.01	1.43	6.17	1.16	105.6	52.8	2.00	61.4	1.72
Mean ± SD	6.99 ± 0.38	5.98 ± 0.93[a]	1.19 ± 0.20	7.05 ± 1.15[a,b]	1.01 ± 0.18[c]	102.2 ± 7.5	54.0 ± 5.5[d]	1.90 ± 0.15	56.8 ± 6.0[d,e]	1.81 ± 0.07[f]
C.V. (%)	5.4	15.6	16.8	16.3	17.8	7.3	10.2	7.9	10.6	3.9

Ratios of k and a for Trolox and α-tocopherol were calculated for the in vitro (k_{Trolox}:$k_{\alpha\text{-TOH}}$ and a_{Trolox}:$a_{\alpha\text{-TOH}}$) and in vivo (Trolox:$k'_{\alpha\text{-TOH}}$ and Trolox:$a'_{\alpha\text{-TOH}}$) results.

[a] Not significantly different from k_{Trolox} in vitro.

[b] Not significantly different from $k_{\alpha\text{-TOH}}$ in vitro.

[c] Not significantly different from k_{Trolox}:$k_{\alpha\text{-TOH}}$ (Student's t-test).

[d] $P < 0.001$ vs. a_{Trolox} in vitro (Student's t-test).

[e] Not significantly different from $a_{\alpha\text{-TOH}}$ in vitro.

[f] Not significantly different from a_{Trolox}:$a_{\alpha\text{-TOH}}$ (Student's t-test).

DISCUSSION

The results of this study show that loading of LDL with Trolox can be used for in vitro experiments that focus on determination of the regression equation variables, k and a, for characterization of the lag time as an index of LDL resistance to oxidation. In a reproducible manner, k and a for α-tocopherol can be predicted from k and a derived from Trolox-loading experiments, which are considerably less time-consuming than equivalent experiments with α-tocopherol. Therefore, these results provide the basis for investigation of larger subject populations within a reasonable time frame.

As also reported by others (2–8), the present study demonstrates that substantial between-subject variability exists in lag times of different individuals. In contrast, maximum rate of oxidation and maximum CD absorbance, two additional indexes of LDL resistance to oxidation, showed much less variability. Loading LDL with α-tocopherol, both in vitro and in vivo, increased LDL α-tocopherol concentrations, as expected. A plateau effect appeared when LDL was loaded with 50 mM α-tocopherol in vitro, and at day 3–6 of supplementation in vivo. While other antioxidant concentrations did not change during in vivo supplementation with α-tocopherol, LDL γ-tocopherol concentrations decreased, following an inverse pattern compared with that of α-tocopherol, confirming previous reports (3, 28). Also, LDL lipid composition did not change during α-tocopherol supplementation. In contrast, the protein content of LDL, expressed as % of total LDL by weight, decreased successively throughout the observation period. Due to standardization of LDL concentrations on cholesterol content (vide supra), changes in protein content of LDL need to be interpreted in relation to LDL cholesterol content. Consequently, the changes observed should be regarded as changes in the protein to lipid ratio of LDL during vitamin E supplementation, the underlying mechanisms of which need to be further explored.

Simultaneously, all three: lag time, time of maximum rate of oxidation, and time of maximum CD absorbance, increased significantly. The increase in LDL α-tocopherol concentrations by ~10 mol α-tocopherol/mol LDL during in vitro loading with 250 mM RRR-α-tocopherol for 3 h was accompanied by a 1.5-fold increase in lag time as compared to an increase in LDL α-tocopherol concentrations by ~8.5 mol/mol that was accompanied by a 1.6-fold increase in lag time during in vivo supplementation with 800–1200 IU/d for 6 days. This finding is consistent with published data (3). Also, loading LDL samples with 10 mol Trolox/mol LDL in vitro caused a 1.8-fold increase in lag time over baseline values. Overall, the average efficacy of Trolox

TABLE 6. Antioxidants in plasma and LDL of five donors (#11–15) and lipid composition of LDL during supplementation with 800–1200 IU/day RRR-α-tocopherol

Antioxidants	Time (days) 0	3	6	10	17	ANOVA[a] P
Plasma (μmol/L)						
α-Tocopherol	19.45 ± 2.45	47.26 ± 11.35	45.06 ± 7.89	49.22 ± 16.10	43.58 ± 8.97	<0.001[b]
γ-Tocopherol	1.11 ± 0.33	0.55 ± 0.11	0.51 ± 0.14	0.63 ± 0.26	0.50 ± 0.10	<0.001[b]
α-Carotene	0.07 ± 0.02	0.08 ± 0.02	0.08 ± 0.02	0.08 ± 0.03	0.07 ± 0.01	n.s.
β-Carotene	0.34 ± 0.13	0.31 ± 0.12	0.34 ± 0.14	0.35 ± 0.15	0.31 ± 0.16	n.s.
Lycopene	0.32 ± 0.19	0.31 ± 0.19	0.34 ± 0.19	0.37 ± 0.18	0.31 ± 0.18	n.s.
Lutein/Zeaxanthin	0.47 ± 0.31	0.48 ± 0.26	0.46 ± 0.28	0.45 ± 0.24	0.52 ± 0.29	n.s.
Canthaxanthin	0.14 ± 0.07	0.15 ± 0.09	0.13 ± 0.06	0.15 ± 0.08	0.14 ± 0.09	n.s.
Cryptoxanthin	0.31 ± 0.29	0.29 ± 0.29	0.29 ± 0.36	0.30 ± 0.32	0.20 ± 0.17	n.s.
LDL (mol/mol)						
α-Tocopherol	5.74 ± 0.62	12.59 ± 1.27	14.19 ± 1.97	12.99 ± 1.52	13.27 ± 1.65	<0.001[b]
γ-Tocopherol	0.29 ± 0.07	0.12 ± 0.03	0.13 ± 0.02	0.12 ± 0.02	0.11 ± 0.02	<0.001[b]
α-Carotene	0.04 ± 0.02	0.04 ± 0.01	0.04 ± 0.01	0.04 ± 0.02	0.04 ± 0.01	n.s.
β-Carotene	0.20 ± 0.07	0.17 ± 0.03	0.21 ± 0.07	0.20 ± 0.05	0.17 ± 0.06	n.s.
Lycopene	0.14 ± 0.07	0.17 ± 0.07	0.16 ± 0.06	0.18 ± 0.07	0.17 ± 0.07	n.s.
Lutein/Zeaxanthin	0.08 ± 0.05	0.08 ± 0.04	0.10 ± 0.05	0.08 ± 0.04	0.10 ± 0.05	n.s.
Canthaxanthin	0.05 ± 0.02	0.06 ± 0.02	0.06 ± 0.03	0.05 ± 0.02	0.05 ± 0.03	n.s.
Cryptoxanthin	0.10 ± 0.10	0.10 ± 0.09	0.11 ± 0.13	0.10 ± 0.12	0.09 ± 0.09	n.s.
LDL composition (weight %)						
Total cholesterol	31.51 ± 1.83	31.24 ± 1.24	30.37 ± 0.88	31.54 ± 0.40	32.11 ± 1.21	n.s.
Triglycerides	6.70 ± 2.46	6.95 ± 1.18	8.46 ± 0.94	7.82 ± 1.01	8.10 ± 1.51	n.s.
Free cholesterol	8.10 ± 3.24	9.47 ± 1.87	9.55 ± 0.36	10.13 ± 0.47	9.12 ± 1.34	n.s.
Cholesteryl ester	39.31 ± 3.79	36.54 ± 2.63	34.94 ± 1.41	35.95 ± 0.89	38.60 ± 2.87	<0.05[c]
Phospholipids	18.52 ± 0.80	19.04 ± 1.31	19.33 ± 0.50	18.27 ± 0.37	18.68 ± 0.67	n.s.
Protein	27.37 ± 1.27	24.03 ± 3.64	22.39 ± 3.58	21.45 ± 5.42	19.96 ± 4.35	<0.05[d]

[a]Repeated measures ANOVA with Tukey multiple comparison test.
[b]Day 0 vs. days 3, 6, 10, and 17.
[c]Day 0 vs. day 6.
[d]Day 0 vs. day 10 and day 0 vs. day 17.

was 1.17 times the efficacy of α-tocopherol, as is evident from the ratio k_{Trolox}:$k_{\alpha\text{-TOH}}$ in Table 4, suggesting that, on a molar basis, a slight advantage for Trolox exists in protecting against the onset of oxidation in LDL.

While loading LDL with α-tocopherol, both in vitro and in vivo, was accompanied by a significant decrease in the maximum rate of oxidation, Trolox did not have such an effect. The lack of effect of Trolox on oxidation rate is clearly evident from the characteristics of the CD absorbance versus time curves in Fig. 1, where no changes in CD formation were observed during the lag time, and all CD absorbance curves were similar in shape during the propagation phase, i.e., regarding the oxidation rate. On the other hand, maximum CD absorbance decreased significantly with increasing Trolox concentrations, but not when LDL was loaded with α-tocopherol. These differences suggest that α-tocopherol and Trolox act differently in protecting LDL against oxidation.

A high between-subject variability of k and a for α-tocopherol had been observed previously (12), and was confirmed by the results of this study. When the data of the 15 subjects of this study were combined, the between-subject CV was 20.6% for $k_{\alpha\text{-TOH}}$ and 20.7% for $a_{\alpha\text{-TOH}}$. In this study, k and a values obtained through loading LDL with Trolox are shown for the first time. They also showed substantial between-subject variability, i.e., CV of 14.3% for k_{Trolox} and 19.6% for a_{Trolox}. However, compared with α-tocopherol, the between-subject variability of the efficacy (k) of Trolox was considerably smaller, perhaps as a result of variability induced, for instance, by between-subject variability in α-tocopherol incorporation into LDL. The overall relatively high variability in k and a is not surprising, because several variables have been shown to affect LDL susceptibility to oxidation (5–11). These variables may both affect the efficacy of α-tocopherol in protecting LDL and, in concert, constitute the α-tocopherol-independent variable a. For investigation of the effects of one or more of these additional variables that contribute to the lag time in general, and to either k and a or both in particular, the approach of obtaining k and a values presented in this study can be recommended.

This study focused on the efficacy of Trolox versus RRR-α-tocopherol in protecting LDL from oxidation in the ex vivo model of copper ion-induced oxidation of

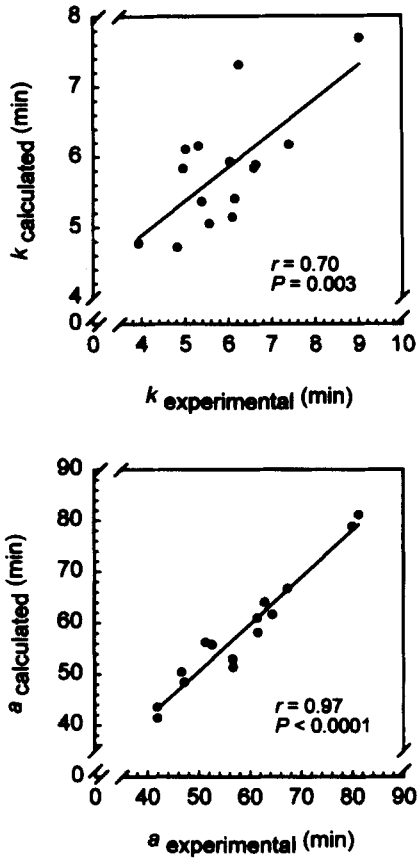

Fig. 2. Correlations between calculated k values, as calculated from k values for Trolox divided by k_{Trolox}:$k_{\alpha\text{-TOH}}$ (from Table 4) and experimental k values for α-tocopherol (from Table 4), are shown in the upper panel and the respective values for a (from Table 4) in the lower panel.

LDL (1). There is still considerable debate about the mechanisms by which LDL is oxidized in vivo and those that trigger both early events and progression of atherogenesis in humans. Particularly due to their complexity, the in vivo conditions may differ substantially from those in this and other ex vivo models currently used (29). While several lines of evidence suggest that transition metal ions, including copper and iron, are involved in oxidation of LDL and atherogenesis (30–32), redundant mechanisms have been proposed, on the other hand, to efficiently chelate free metal ions in vivo, rendering them redox inactive (33). Recently, thiol compounds have been shown to further mediate metal ion-dependent oxidation of LDL (34). However, model systems that allow optimal simulation of the in vivo situation in humans are still not available. In line with these limitations, the data presented here do not preclude that the efficacy of α-tocopherol (k) and other antioxidants that constitute the α-tocopherol-independent variable (a) are similar in the in vivo situation. Also, it remains to be investigated whether or not these antioxidants are equally effective in other types of oxidative stress, such as cell-mediated oxidation of LDL.

This work was supported by the Austrian Science Foundation (grants S07102-MED and P11690-MED) and the Association for International Cancer Research, U.K. (grant 96-10).

Manuscript received 23 June 1997 and in revised form 2 September 1997.

REFERENCES

1. Esterbauer, H., G. Striegl, H. Puhl, and M. Rotheneder. 1989. Continuous monitoring of in vitro oxidation of human low density lipoprotein. *Free Radical Res. Commun.* **6:** 67–75.
2. Jialal, I., and S. M. Grundy. 1992. Effect of dietary supplementation with alpha-tocopherol on the oxidative modification of low density lipoprotein. *J. Lipid Res.* **33:** 899–906.
3. Dieber-Rotheneder, M., H. Puhl, G. Waeg, G. Striegl, and H. Esterbauer. 1991. Effect of oral supplementation with D-α-tocopherol on the vitamin E content of human low density lipoproteins and resistance to oxidation. *J. Lipid Res.* **32:** 1325–1332.
4. Princen, H. M. G., G. van Poppel, C. Vogelezang, R. Buytenhek, and F. J. Kok. 1992. Supplementation with vitamin E but not β-carotene in vivo protects low density lipoprotein from lipid peroxidation in vitro. Effect of cigarette smoking. *Arterioscler. Thromb.* **12:** 554–562.
5. Esterbauer, H., G. Striegl, H. Puhl, S. Oberreither, M. Rotheneder, M. El-Saadani, and G. Jürgens. 1989. The role of vitamin E and carotenoids in preventing oxidation of low density lipoproteins. *Annu. NY Acad. Sci.* **570:** 254–267.
6. Jialal, I., E. P. Norkus, L. Cristol, and S. C. Grundy. 1991. β-Carotene inhibits the oxidative modification of low density lipoprotein. *Biochim. Biophys. Acta.* **1086:** 134–138.
7. Reaven, P., S. Parthasarathy, B. J. Grasse, E. Miller, D. Steinberg, and J. L. Witztum. 1993. Effects of oleate-rich and linoleate-rich diets on the susceptibility of low density lipoprotein to oxidative modification in mildly hypercholesterolemic subjects. *J. Clin. Invest.* **91:** 668–676.
8. Kleinveld, H. A., A. H. J. Naber, A. F. H. Stalenhoef, and P. N. M. Demacker. 1993. Oxidation resistance, oxidation rate, and extent of oxidation of human low-density lipoprotein depend on the ratio of oleic acid content to linoleic acid content: studies in vitamin E-deficient subjects. *Free Radical Biol. Med.* **15:** 273-280.
9. Stocker, R., V. W. Bowry, and B. Frei. 1991. Ubiquinol-10 protects human low density lipoprotein more efficiently against lipid peroxidation than does α-tocopherol. *Proc. Natl. Acad. Sci. USA.* **88:** 1646–1650.
10. Stalenhoef, A. F. H., J. C. Defesche, H. A. Kleinfeld, P. N. M. Demacker, and J. J. P. Kastelein. 1994. Decreased resistance against in vitro oxidation of LDL from patients with familial defective apolipoprotein B-100. *Arterioscler. Thromb.* **14:** 489–493.
11. De Graaf, J., H. L. M. Hak-Lemmers, M. P. C. Hectors, P. N. M. Demacker, J. C. M. Hendriks, and A. F. H. Stalenhoef. 1991. Enhanced susceptibility to in vitro oxidation

of the dense low density lipoprotein subfraction in healthy subjects. *Arterioscler. Thromb.* **11:** 298–306.
12. Esterbauer, H., J. Gebicki, H. Puhl, and G. Jürgens. 1992. The role of lipid peroxidation and antioxidants in oxidative modification of LDL. *Free Radical Biol. Med.* **13:** 341–390.
13. Esterbauer, H., H. Puhl, G. Waeg, A. Krebs, and M. Dieber-Rotheneder. 1992. The role of vitamin E in lipoprotein oxidation. *In* Vitamin E in Health and Disease. L. Packer and J. Fuchs, editors. Marcel Dekker, New York. 649–671.
14. Esterbauer, H., M. Dieber-Rotheneder, G. Striegl, and G. Waeg. 1991. Role of vitamin E in preventing the oxidation of low-density lipoprotein. *Am. J. Clin. Nutr.* **53:** 314S–321S.
15. Scott, J. W., W. M. Cort, H. Harley, D. R. Parrish, and G. Saucy. 1974. 6-Hydroxychroman-2-carboxylic acids: novel antioxidants. *J. Am. Oil Chem. Soc.* **51:** 200–203.
16. Wu, T. W., N. Hashimoto, J. Wu, D. Carey, R. K. Li, D. A. G. Mickle, and R. D. Weisel. 1990. The cytoprotective effect of Trolox demonstrated with three types of human cells. *Biochem. Cell Biol.* **68:** 1189–1194.
17. Barclay, L. R. C., S. J. Locke, and J. M. MacNeil. 1985. Autooxidation in micelles. Synergism of vitamin C with lipid-soluble vitamin E and water-soluble Trolox. *Can. J. Chem.* **63:** 366–374.
18. Suttorp, N., W. Toepfer, and L. Roka. 1986. Antioxidant defense mechanisms of endothelial cells: glutathione redox cycle versus catalase. *Am. J. Physiol.* **251:** C671–680.
19. Kögl, C., W. Schneider, and E. F. Elstner. 1994. The influence of magnesium-pyridoxal-5′-phosphate-glutamate in comparison with probucol, α-tocopherol and trolox on copper-induced oxidation of human low density lipoprotein in vitro. *Biochem. Pharmacol.* **47:** 2201–2206.
20. Zeng, L. H., J. Wu, D. Carey, and T. W. Wu. 1991. Trolox and ascorbate: are they synergistic in protecting liver cells in vitro and in vivo? *Biochem. Cell Biol.* **69:** 198–201.
21. Rubinstein, J. D., E. J. Lesnefsky, R. M. Byler, P. V. Fennessey, and L. D. Horwitz. 1992. Trolox C, a lipid-soluble membrane protective agent, attenuates myocardial injury from ischemia and reperfusion. *Free Radical Biol. Med.* **13:** 627–634.
22. Penn, J. S., B. L. Tolman, and L. E. Bullard. 1997. Effect of a water-soluble vitamin E analog, Trolox C, on retinal vascular development in an animal model of retinopathy of prematurity. *Free Radical Biol. Med.* **22:** 977–984.
23. Kleinveld, H. A., H. L. M. Hak-Lemmers, A. F. H. Stalenhoef, and P. N. M. Demacker. 1992. Improved measurement of low-density-lipoprotein susceptibility to copper-induced oxidation: application of a short procedure for isolating low-density-lipoprotein. *Clin. Chem.* **38:** 2066–2072.
24. Ramos, P., S. P. Gieseg, B. Schuster, and H. Esterbauer. 1995. Effect of temperature and phase transition on oxidation resistance of low density lipoprotein. *J. Lipid. Res.* **36:** 2113–2128.
25. Gieseg, S. P., and H. Esterbauer. 1994. Low density lipoprotein is saturable by pro-oxidant copper. *FEBS Lett.* **343:** 188–194.
26. Ziouzenkova, O., B. M. Winklhofer-Roob, H. Puhl, J. M. Roob, and H. Esterbauer. 1996. Lack of correlation between the α-tocopherol content of plasma and LDL, but high correlations for γ-tocopherol and carotenoids. *J. Lipid. Res.* **37:** 1936–1946.
27. Puhl, H., G. Waeg, and H. Esterbauer. 1994. Methods to determine oxidation of low density lipoproteins. *Methods Enzymol.* **233:** 425–441.
28. Winklhofer-Roob, B. M., O. Ziouzenkova, H. Puhl, H. Ellemunter, P. Greiner, G. Müller, M. A. van't Hof, H. Esterbauer, and D. H. Shmerling. 1995. Impaired resistance to oxidation of low density lipoprotein in cystic fibrosis. Improvement during vitamin E supplementation. *Free Radical Biol. Med.* **19:** 725–733.
29. Berliner, J. A., and J. W. Heinecke. 1996. The role of oxidized lipoproteins in atherogenesis. *Free Radical Biol. Med.* **20:** 707–727.
30. Steinbrecher, U. P., S. Parthasarathy, D. S. Leake, J. L. Witzum, and D. Steinberg. 1984. Modification of low density lipoprotein by endothelial cells involves lipid peroxidation and degradation of low density lipoprotein phospholipids. *Proc. Natl. Acad. Sci. USA.* **81:** 3883–3887.
31. Smith, C., M. J. Mitchinson, O. I. Aruoma, and B. Halliwell. 1992. Stimulation of lipid peroxidation and hydroxyl-radical generation by the contents of human atherosclerotic lesions. *Biochem. J.* **286:** 901–905.
32. Dubick, M. A., G. C. Hunter, S. M. Casey, and C. L. Keen. 1987. Aortic ascorbic acid, trace elements, and superoxide dismutase activity in human aneurysmal and occlusive disease. *Proc. Soc. Exp. Biol. Med.* **184:** 138–143.
33. Dabbagh, A. J., and B. Frei. 1995. Human suction blister interstitial fluid prevents metal ion-dependent oxidation of low density lipoprotein by macrophages and in cell-free systems. *J. Clin. Invest.* **96:** 1958–1966.
34. Lynch, S. M., and B. Frei. 1997. Physiological thiol compounds exert pro- and anti-oxidant effects, respectively, on iron- and copper-dependent oxidation of human low-density lipoprotein. *Biochim. Biophys. Acta.* **1345:** 215–221.