FEBS Letters 365 (1995) 164–166 FEBS 15520

Total antioxidant activity of low density lipoproteins and the relationship with α-tocopherol status

Nicholas J. Miller[a], George Paganga[a], Sheila Wiseman[c], Wim Van Nielen[c], Lilian Tijburg[c], Philip Chowienczyk[b], Catherine A. Rice-Evans[a,*]

[a]*Free Radical Research Group, UMDS - Guy's Hospital, St Thomas's Street, London SE1 9RT, UK*
[b]*Department of Clinical Pharmacology, UMDS - Guy's Hospital, St Thomas's Street, London SE1 9RT, UK*
[c]*Unilever Research Laboratorium, Vlaardingen, The Netherlands*

Received 13 April 1995; revised version received 19 April 1995

Abstract A rapid method is described for measuring the antioxidant activity of low density lipoproteins. Studies were undertaken on individuals attending a hyperlipidaemia clinic, an unsupplemented group and a group after supplementation with 300 mg dl-α-tocopherol acetate for nine weeks. The results show a positive correlation between the antioxidant activity and α-tocopherol content of LDL in the supplemented group.

Key words: Antioxidant activity; Low density lipoprotein; ABTS$^{\bullet+}$; Vitamin E

1. Introduction

The importance of antioxidants in the maintenance of health and protection from disease is becoming increasingly well-recognised [1] and in particular the levels of individual antioxidant nutrients required for optimal health [2]. Several methods have been developed recently for monitoring the total antioxidant activity (i.e. the total collective potential of the constituent antioxidants to scavenge radicals) of plasma [3–7], saliva [8], pure antioxidant compounds [9], food extracts and beverages [10–12]. The methods are based on the composite hydrogen-donating abilities of the antioxidants in the matrix in question to scavenge generated radicals to an extent and on a timescale dependent on their total antioxidant potential. However, a major question where body fluids are concerned is the contribution from unidentified antioxidants. In addition it is not clear to what extent the lipid-soluble antioxidants in plasma, especially α-tocopherol and the carotenoids, are available and accessible to participate in the reaction in methods not involving generation of peroxyl radicals.

In this work a rapid, sensitive method for assessing the total antioxidant activity of LDL is described based on its interaction with the ABTS$^{\bullet+}$ [2,2′-azinobis(3-ethylbenzothiazoline-6-sulphonic acid)] radical cation, an application of the previously established method for plasma, saliva and beverages [5,8,12]. The results show that the antioxidant activity of LDL correlates with the α-tocopherol content, $r = 0.74$, the intercept presumably indicating the contribution from other lipophilic antioxidants.

2. Materials and methods

Reagents for total antioxidant activity were prepared as previously described [5]. Trolox (Hoffman-La Roche) (2.5 mM in buffer, pH 7.4) was used as a stock standard from which analytical standards were freshly prepared as required. Working solutions of hydrogen peroxide were freshly prepared after initial dilution to a concentration of 500 mM. Aristar hydrogen peroxide is supplied as a 30% solution, with a specific gravity of 1.10: a 500 mM solution was therefore prepared by diluting 515 μl to 10 ml and the concentration checked spectroscopically ($\varepsilon_{mM} = 39.4$ at 240 nm) [13]. The assay was carried out using a Cobas Fara centrifugal analyser (Roche Diagnostic Systems SA). In the protocol devised 250 μl of mixed ABTS/myoglobin reagent in buffer was mixed with 50 μl of Trolox standard or LDL preparation (1.13–4.52 mg/ml LDL protein), the pipettor probe flushed with 20 μl of water, then 20 μl of 3.125 mM hydrogen peroxide (followed by 10 μl of water to flush the probe) added to start the reaction. The incubation volume was thus 350 μl; reagents were prepared so that on dilution into this incubation volume they were at the desired final concentration (4.36 μM metmyoglobin, 436 μM ABTS, 180 μM H_2O_2).

Using these reagent concentrations and a temperature of 30°C the end of the lag phase for the highest Trolox standard (100 μM initial concentration, 14.30 μM final concentration) was 248 s after the addition of H_2O_2; a 248-s period between the initial and final readings was used as the measuring time for the assay. A dose-response curve was derived using a logit/log 4 plot of absorbance at 734 nm against a range of Trolox standards (0, 2.86, 5.72, 8.58, 11.44 and 14.30 μM final concentrations).

The method is based on the ability of Trolox, or antioxidants within the LDL, to scavenge the ABTS$^{\bullet+}$ radical cation, as detected by a decrease in absorbance at 734 nm. There is no interfering effect of the antioxidants on ABTS$^{\bullet+}$ production, as evidenced by the fact that the assay can also be carried out by allowing antioxidant solutions to decolorise the pre-formed ABTS$^{\bullet+}$ (unpublished data). The use of an absorbance maximum in the near-IR region of the spectrum (734 nm) is an important feature of the assay in that sample turbidity is not a confounding factor at these wavelengths; LDL preparations of up to 5 mg/ml concentration have been assayed.

29 blood samples were obtained from patients attending a lipid clinic. 8 samples were obtained from patients who had not taken α-tocopherol supplements and 21 from patients supplemented with 300 mg dl-α-tocopherol acetate daily for 9 weeks. Approval for the project was given by the Ethics Committee of the West Lambeth Health Authority. Blood was taken into acid citrate-dextrose as an anticoagulant and LDL was prepared from the plasma by density gradient ultracentrifugation [14]. The protein content of the LDL preparations was measured by the method of Markwell et al. [15]; values for antioxidant activity and for concentration of α-tocopherol in LDL were standardised to protein content. LDL α-tocopherol was measured by normal phase HPLC, using δ-tocopherol as an internal standard [16]. Cholesterol was measured by the cholesterol esterase/cholesterol oxidase method (Boehringer Mannheim GmbH) [17].

The statistical significance of the degree of association between LDL total antioxidant activity (LDL TAA) and other measurements was assessed by deriving the Spearman rank-order correlation coefficient.

3. Results

The measured values for LDL total antioxidant activity (LDL TAA), α-tocopherol, cholesterol and α-tocopherol/cholesterol ratio on 29 LDL preparations are shown in

*Corresponding author. Fax: (44) (171) 955 4983.

0014-5793/95/$9.50 © 1995 Federation of European Biochemical Societies. All rights reserved.
SSDI 0014-5793(95)00448-3

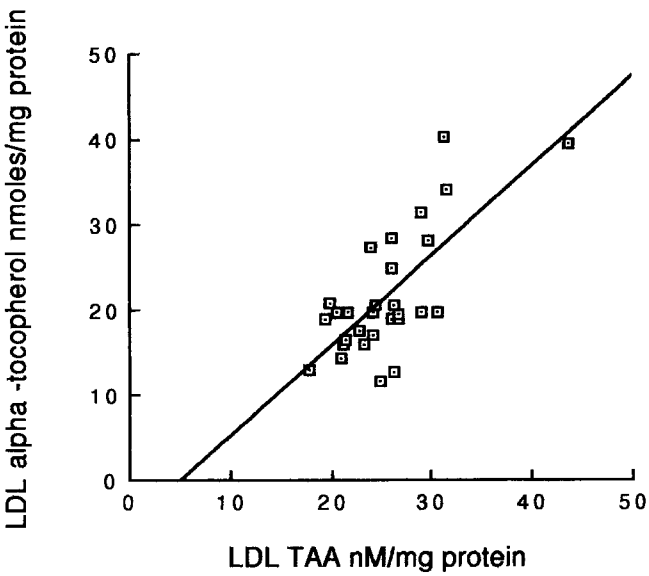

Fig. 1. Plot of matched LDL TAA values (nM/mg protein) against LDL α-tocopherol concentrations (nmol/mg protein), $n = 29$, $P < 0.05$.

Table 1, and separated into results from subjects not taking α-tocopherol supplements in Table 2 and those from subjects taking α-tocopherol supplements in Table 3. All the results shown are expressed per mg of LDL protein to allow for variations in the lipoprotein concentration of these preparations. Matched plasma α-tocopherol, plasma cholesterol concentrations and plasma α-tocopherol/cholesterol ratios are also shown. In order to investigate the association between α-tocopherol levels and measures of the total antioxidant activity of LDL, hyperlipidaemic patients were studied with α-tocopherol levels ranging from 37.3–54.9 (unsupplemented group) and 35.8–151.6 (supplemented) (Tables 2 and 3) giving LDL α-tocopherol ranges of 11.6–19.7 (nmol/mg protein) (unsupplemented) and 16.0– 47.9 (nmol/mg protein), respectively.

No statistically significant association was observed between the LDL TAA and the other parameters measured in the non-supplemented group (using the Spearman rank-order correlation coefficient) (Table 2). However, the LDL TAA correlated well with both α-tocopherol content and also α-tocopherol/cholesterol ratio in specimens from individuals who were supplementing with α-tocopherol (Table 3). There was no negative (or positive) correlation between LDL TAA and LDL cholesterol. There was also a good correlation between LDL total antioxidant activity and plasma α-tocopherol and α-tocopherol/cholesterol ratio; the fact that a higher degree of statistical significance was observed between LDL TAA and plasma α-tocopherol can be interpreted as a reflection of the better precision of the plasma measurement, involving, as it does, fewer analytical steps and higher α-tocopherol values than the equivalent measurements in LDL.

The correlation between the LDL TAA and the α-tocopherol concentration in LDL of all subjects is shown in Fig. 1, $r = 0.74$, with an intercept on the TAA axis of 5.2 nanomolar of antioxidant activity/mg protein.

4. Discussion

A novel spectrophotometric method is presented for the direct measurement of the total antioxidant activity of LDL. In this method the ABTS radical monocation ($ABTS^{\bullet+}$) is generated in the aqueous phase of the analytical mixture to which LDL is added. Nevertheless antioxidants in lipoprotein particles were demonstrated to be capable of suppressing its formation, although the extent to which all the minor antioxidants participate is unclear. The LDL TAA correlates well with the α-tocopherol content of the LDL in the supplemented group, the correlation coefficient for all samples being $r = 0.74$. The intercept value presumably reflects the contribution from non-α-tocopherol antioxidants to the antioxidant status of the LDL including γ-tocopherol, carotenoids, ubiquinol and possibly flavonoids [18,19]. Since α-tocopherol and Trolox, the analytical standard used in the assay, have the same antioxidant activity in this system [5], a direct comparison of the two measurements is valid. Previously a method for antioxidant capacity in terms of the ability of LDL to inhibit peroxyl radical-dependent oxidation of luminol has been reported [20]. These authors applied Trolox as standard and based the calculation on the assumption that Trolox is capable of scavenging 2 peroxyl radicals per mole in LDL [3]. The factor of 2 difference between the gradient of the slope derived from their association between antioxidant activity and α-tocopherol content of LDL, and that described here is explained by the application of a 1:1 stoichiometry of Trolox and the $ABTS^{\bullet+}$ radical in this system. Kagan et al. [21] reported mean LDL α-tocopherol concentrations of 23.0 ± 2.0 nmol/mg LDL protein, which is very close to the mean value of 21.6 ± 1.5 nmol/mg protein detected here: whilst other

Table 1
LDL antioxidant activity, LDL α-tocopherol, LDL cholesterol, LDL α-tocopherol/cholesterol ratio, plasma α-tocopherol, plasma cholesterol and plasma α-tocopherol/cholesterol ratio in plasma

	Mean ± S.D.	Range
LDL TAA (nM/mg protein)	24.7 ± 3.7	17.7–31.4
LDL α-tocopherol (nmol/mg protein)	21.8 ± 8.2**	11.6–47.9
LDL cholesterol (mg/mg protein)	1.40 ± 0.22	0.94–1.92
LDL α-tocopherol/cholesterol ratio	15.5 ± 5.3**	7.7–31.3
Plasma α-tocopherol (μmol/l)	55.4 ± 23.0***	35.8–151.6
Plasma cholesterol (mmol/l)	6.00 ± 0.87	4.40–7.80
Plasma α-tocopherol/cholesterol ratio	9.3 ± 3.9***	5.7–25.4

$n = 29$, mean ± S.D. shown. All LDL results are expressed as 'per mg of LDL protein'. Correlation with LDL TAA (nM/mg protein) $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.005$ (Spearman rank-order correlation coefficient).

Table 2
LDL antioxidant activity, LDL α-tocopherol, LDL cholesterol, LDL α-tocopherol/cholesterol ratio, plasma α-tocopherol, plasma cholesterol and plasma α-tocopherol/cholesterol ratio in plasma from subjects not taking α-tocopherol supplements

	Mean ± S.D.	Range
LDL TAA (nM/mg protein)	22.6 ± 2.8	17.7–26.3
LDL α-tocopherol (nmol/mg protein)	15.1 ± 2.7	11.6–19.7
LDL cholesterol (mg/mg protein)	1.39 ± 0.23	0.94–1.66
LDL α-tocopherol/cholesterol ratio	11.0 ± 2.1	7.7–13.7
Plasma α-tocopherol (μmol/l)	45.1 ± 6.5	37.3–54.9
Plasma cholesterol (mmol/l)	6.03 ± 0.79	4.95–7.71
Plasma α-tocopherol/cholesterol ratio	7.6 ± 1.7	5.9–11.1

$n = 8$, mean ± S.D. shown. All LDL results are expressed as 'per mg of LDL protein'.

Table 3
LDL antioxidant activity, LDL α-tocopherol, LDL cholesterol, LDL α-tocopherol/cholesterol ratio, plasma α-tocopherol, plasma cholesterol and plasma α-tocopherol/cholesterol ratio in plasma from subjects supplementing with α-tocopherol

	Mean ± S.D.	Range
LDL TAA (nM/mg protein)	25.5 ± 3.7[1]	19.4–31.4
LDL α-tocopherol (nmol/mg protein)	24.4 ± 8.2[1,*]	16.0–47.9
LDL cholesterol (mg/mg protein)	1.43 ± 0.21	1.11–1.92
LDL α-tocopherol/cholesterol ratio	17.2 ± 5.1*	10.1–31.3
Plasma α-tocopherol (μmol/l)	59.3 ± 25.9***	35.8–151.6
Plasma cholesterol (mmol/l)	6.00 ± 0.92	4.40–7.80
Plasma α-tocopherol/cholesterol ratio	10.0 ± 4.2**	5.7–25.4

$n = 21$, mean ± S.D. shown. All LDL results are expressed as 'per mg of LDL protein'. Correlation with LDL TAA (nM/mg protein) $*P < 0.05$, $**$ $P < 0.01$, $***P < 0.005$ (Spearman rank-order correlation coefficient).
[1] $*P < 0.05$ supplemented vs. non-supplemented (Table 2) (Student's t-test).

authors have detected somewhat lower levels of α-tocopherol in LDL of non-supplemented normal individuals (11.58 ± 3.34 nmol/mg protein [22]). The higher levels presented here reflect the increased lipoprotein levels of α-tocopherol anticipated in plasma lipoproteins derived from hyperlipidaemic patients [23,24].

The findings in this study support those of Esterbauer et al. [18] who showed a correlation between α-tocopherol content of LDL and its oxidation resistance (measured by lag phase to copper-induced oxidation) from normal, healthy individuals supplementing with RRR α-tocopherol ($r = 0.715$), but not in non-supplemented volunteers, although the given effectiveness of α-tocopherol to increase oxidation resistance varied from person to person.

The method described here will be of use in the in vitro investigation of factors affecting LDL oxidation, as well as in supplementation studies designed to assess the absorption and efficacy of lipophilic antioxidants.

Acknowledgements: The financial support of the Ministry of Agriculture, Fisheries and Food and of the British Heart Foundation is acknowledged.

References

[1] Halliwell, B. (1995) in: Free Radicals and Oxidative Stress, Environment, Drugs and Food Additives (C. Rice-Evans and B. Halliwell, Eds.) Portland Press, London, in press.
[2] Gey, K.F. (1994) in: Free Radicals in the Environment, Medicine and Toxicology (H. Nohl, H. Esterbauer and C. Rice-Evans, Eds.) Richelieu Press, London, pp 181–219.
[3] Wayner, D.D.M., Burton, G.W., Ingold, K.U., Barclay, L.R.C. and Locke, S.J. (1987) Biochim. Biophys. Acta 924, 408–419.
[4] Whitehead, T.P., Thorpe, G.H.G. and Maxwell, S.R.J. (1992) Anal. Chim. Acta 266, 265–277.
[5] Miller, N.J., Rice-Evans, C.A., Davies, M.J., Gopinathan, V. and Milner, A., (1993) Clin. Sci. 84, 407–412.
[6] Rice-Evans, C. and Miller, N.J. (1994) Methods Enzymol. 234, 279–293.
[7] Ghiselli, A., Serafini, M., Maiani, G., Azzini, E. and Ferro-Lazzi, A. (1995) Free Rad. Biol. Med. 18, 29–36.
[8] Moore, S., Calder, K.A.C., Miller, N.J. and Rice-Evans, C.A. (1994) Free Rad. Res. 21, 417–425.
[9] Rice-Evans, C.A., Miller, N.J., Bolwell, P.G., Bramley, P.M. and Pridham, J.B. (1995) Free Rad. Res. 22.
[10] Maxwell, S., Cruickshank, A. and Thorpe, G. (1994) Lancet 344, 194.
[11] Serafini, M., Ghiselli, A. and Ferro-Luzzi, A. (1994) Lancet 344, 626.
[12] Miller, N., Diplock, A.T. and Rice-Evans, C. (1995) J. Agricult. Food Chem., in press.
[13] Bergmeyer, H.U. (1983) Methods Enzym. Anal. 3, pp. 277.
[14] Chung, B.H., Wildinson, T., Geer, J.C. and Segrest, J.P. (1980) J. Lipid Res. 21, 284–291.
[15] Markwell, M.A.K., Haas, S.M., Bieber, L.L. and Tolbert, N.E. (1978) Anal. Biochem. 87, 206–210.
[16] Rice-Evans, C.A., Diplock, A.T. and Symons, M.C.R. (1991) in: Laboratory Techniques in Biochemistry and Molecular Biology (Burdon, R.H. and van Knippenberg, P.H., Eds.) Elsevier, Amsterdam, pp. 185–191.
[17] Siedel, J., Hagele, E.O., Ziegenhorn, J. and Wahlefeld, A.W. (1983) Clin. Chem. 29, 1075–1080.
[18] Esterbauer, H., Dieber-Rotheneder, M., Waeg, G., Puhl, H. and Tatzber, F. (1990) Biochem. Soc. Trans. 18, 1059–1061.
[19] Stocker, R., Bowry, V.W. and Frei, B. (1991) Proc. Natl. Acad. Sci. USA 88, 1646–1650.
[20] Smith, D., O'Learly, V.J. and Darley-Usmar, V.M. (1993) Biochem. Pharmacol. 45, 2195–2201.
[21] Kagan, V.E., Serbinova, E.A., Forte, T., Scita, G. and Packer, L. (1992) J. Lipid Res. 33, 385–397.
[22] Esterbauer, H., Gebicki, J., Puhl, H. and Jurgens, G. (1992) Free Rad. Biol. Med. 13, 341–390.
[23] Bieri, J.G., Poukka, R. and Thorp, S. (1977) Am. J. Clin. Nutr. 40, 747–751.
[24] Sokol, R.J., Balistreri, W.F., Hoofnagle, J.H. and Jones, E.A. (1985) Am. J. Clin. Nutr. 41, 66–72.