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THE ROLE OF α-TOCOPHEROL AS A PEROXYL RADICAL SCAVENGER IN HUMAN LOW DENSITY LIPOPROTEIN

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Abstract—It is thought that the oxidation of low density lipoprotein (LDL) plays a key role in the pathogenesis of atherosclerosis. It is well known that lipid peroxidation reactions are propagated by peroxyl radicals and it follows, therefore, that the capacity of an individual LDL particle to scavenge these oxidants may be an important indicator of its atherogenic potential. There are several components within LDL which scavenge peroxyl radicals including chain breaking antioxidants and amino acids on the protein. It is not clear at present which of these antioxidants is most important. In attempting to address the question we have used a simple method for the measurement of the total capacity of the LDL particle to scavenge peroxyl radicals. This assay depends upon the ability of antioxidants in LDL to inhibit the peroxyl radical dependent oxidation of luminol. We have found that approximately 80% of the antioxidant capacity of LDL, isolated from a number of donors, could be accounted for by the α-tocopherol present in the samples. We have compared these results with those obtained when the identical samples of LDL were oxidized with copper and found, as reported by others, a wide range in the susceptibility of the different LDL preparations to oxidation by this transition metal. We suggest that this variability is unlikely to be due to differences in the ability of an LDL particle to scavenge peroxyl radicals.

One of the risk factors that predispose individuals to coronary heart disease is an elevated level of plasma low density lipoprotein (LDL†) cholesterol [1]. In addition, epidemiological studies have shown that, in European populations, a low concentration of plasma antioxidants, particularly α-tocopherol, increases the risk of developing coronary heart disease [2]. It is known that native LDL is not atherogenic but may be modified in the artery wall to an atherogenic particle. Several possible mechanisms have been suggested which could result in LDL modification *in vivo* [3–5] and evidence is accumulating which favours the idea that the oxidation of the lipid phase of the particle can lead to the formation of a potentially atherogenic conformation of LDL [6, 7]. Lipid oxidation reactions have been investigated in depth over recent years and it has been shown that lipid derived peroxyl radicals play a major role in propagating the oxidative reaction and this also appears to be the case with LDL [6–8]. The role of LDL oxidation in atherosclerosis is consistent with the epidemiological studies since a key factor controlling the rate of LDL oxidation is its content of peroxyl radical scavengers. Indeed, it has been shown that dietary supplementation with the antioxidant α-tocopherol increases the resistance of LDL to oxidation promoted by copper [8–10].

Interestingly, the oxidizability of LDL samples shows considerable donor dependent variation which does not correlate with the endogenous α-tocopherol content of the LDL particle [8–11]. Furthermore, the efficacy of α-tocopherol, either when taken orally or incorporated into LDL *ex vivo*, shows a similar donor dependent variation in inhibiting copper dependent LDL oxidation [8]. Although the precise details of the copper dependent oxidation of LDL remain unclear, a dramatic increase in lipid peroxides is observed during the reaction implicating a key role of peroxyl radicals early in this process [12]. Several factors could explain the lack of a correlation between the α-tocopherol content of LDL and oxidizability measured in this assay and many are currently under investigation [13–16]. For example, although this molecule appears to constitute approximately 80% of the total concentration of known lipid soluble peroxyl radical scavengers in LDL it does not take into account the possibility that the apo B protein could react with lipid derived radicals and terminate propagation of the lipid peroxidation reaction [17, 18]. It has recently been shown that the copper dependent oxidation of LDL requires the presence of endogenous lipid hydroperoxides in the LDL particle and an increase in their concentration greatly increases the rate of oxidation [15, 16]. It is possible that the endogenous lipid hydroperoxide content may vary between individuals. Further possibilities are: (a) variations in fatty acid composition of LDL samples and (b) the presence of other antioxidants (peroxyl radical scavengers), such as ubiquinol, within the molecule [13, 14].

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† Abbreviations: LDL, low density lipoprotein; DTPA, diethylene-triaminepenta-acetic acid; ABAP, 2,2′-azobis-(2-amidinopropane)HCl; trolox, 6-hydroxy-2,5,7,8-tetramethyl-chroman-2-carboxylic acid; 13-HPODE, (9*Z*,11*E*, 13(*S*))-13-hydroperoxyoctadecadien-1-oic acid; TRAP, total (peroxyl) radical trapping antioxidant parameter; PBS, calcium, magnesium free phosphate buffered saline.

In order to determine the relative contribution of α-tocopherol to the peroxyl radical scavenging activity within the LDL particle an experimental system is needed which is insensitive to the lipid hydroperoxide and fatty acid composition of LDL isolated from different donors. Wayner and co-workers [18] have described an assay which could possess these properties for the measurement of the antioxidant status of human plasma samples. Using thermolabile azo compounds, such as 2,2'-azobis(amidinopropane)HCl (ABAP) to initiate the oxidation of plasma they defined total (peroxyl) radical trapping antioxidant parameter (TRAP) and demonstrated that a number of antioxidants such as ascorbate, urate, α-tocopherol and protein all contribute to the antioxidant status of human plasma [18]. We have used a modified form of this assay in which the ABAP dependent oxidation of linoleic acid is used to assess the relative ability of compounds to act as peroxyl radical scavengers [19]. In this system peroxyl radicals for the initiation of peroxidation are derived directly from ABAP and do not require the transition metal dependent decomposition of pre-formed peroxides [19]. In these methods the consumption of oxygen is used to follow the peroxidation reaction and this places limitations on the throughput of the assay since it takes up to 2 hr to measure the TRAP value for one sample [18]. Recently, it has been shown that peroxyl radicals cause the oxidation of luminol to give a chemiluminescent signal which can be monitored in a commercially available luminometer and this could provide an alternative method for the determination of TRAP [20]. In addition it has been shown that the treatment of human LDL with ABAP results in lipid peroxidation [21]. We report here the application of this assay to LDL in order to assess the relative importance of α-tocopherol as a peroxyl radical scavenger in the lipoprotein. In addition we have compared these results with those obtained for the same samples of LDL when oxidized with copper.

MATERIALS AND METHODS

Isolation and characterization of LDL. Human LDL was isolated from the plasma of healthy donors (aged between 20 and 50 years) by differential centrifugation using the method described previously [22]. Some samples of LDL were supplemented with α-tocopherol using the protocol described in Ref. 8. In brief, human plasma, isolated from individual donors was supplemented with α-tocopherol to give a range of concentrations (0.3–1.2 mM final concentration in 1% ethanol v/v) and the LDL isolated from the plasma as described in Ref. 22. After dialysis against calcium, magnesium free phosphate buffered saline (PBS) containing 10 μM EDTA the LDL was sterilized by filtration through a 0.2 μm pore size filter and stored at 4° before use. The LDL protein concentration was determined using bovine serum albumin as a standard and the BCA protein assay reagent (Pierce). Oxidizability of LDL samples in the presence of copper was determined spectrophotometrically as described previously and the time taken to achieve half the maximum absorbance at the end of the propagation phase determined [23]. This parameter is directly proportional to a "lag phase" but is less dependent upon the relative rates of oxidation in the initial propagation phases of the peroxidation reaction which, in our experience, can show donor dependent variation. For each preparation of LDL oxidizability was determined as a function of copper concentration, typically 5–50 μM, and in all cases was found to be maximal at copper concentrations above 25 μM. Concentrations of α-tocopherol in LDL samples were determined as described in Ref 9. Some LDL preparations were supplemented with (9*Z*,11*E*, 13(*S*))-13-hydroperoxyoctadecadien-1-oic acid (13-HPODE) prior to oxidation as described [16].

Assay method. The conditions for the assay were similar to those described in Ref. 20. The assay mixture contained air-saturated buffer; sodium phosphate (100 mM), NaCl (150 mM) pH 7.6, ABAP (7 mM), luminol (0.87 mM), sodium linoleate (7 mM) and diethylene-triaminepenta-acetic acid (DTPA) (10 μM). Sodium linoleate was used without further purification and contained little or no contaminating peroxides as measured by HPLC using the column described in Ref. 9 and a mobile phase composed of 800:10:0.8, hexane:isopropanol:acetic acid (result not shown). The assay mixture was pre-incubated for 1–1.5 hr at 37° in the dark prior to the start of the experiment before being dispensed into the sample tubes for the luminometer (LKB model 1251). This procedure resulted in stable signals in the instrument. Under these conditions eight samples, each containing 575 μL of assay mixture, could be monitored during one experiment. The chemiluminescence of each sample was measured at 1-min intervals over a period of 5–10 min after which samples of LDL (1–2 mg protein/mL typically 30–100 μL) or α-tocopherol (0.5–2 nmol) were added at 15-sec intervals and the chemiluminescence measured again at 1-min intervals. The addition of either α-tocopherol or LDL resulted in a temporary inhibition of the signal. The length of this inhibition period or "lag phase" (T) was estimated as shown in Fig. 1. After a stable and maximal signal had again been achieved an aliquot of the antioxidant 6-hydroxy-2,5,7,8-tetramethyl-chroman-2-carboxylic acid (trolox), (1 nmol), was added to act as an internal standard for peroxyl radical scavenging activity [18]. When present trolox and α-tocopherol were added as ethanolic solutions (ethanol 1% v/v). The lag phase determined with trolox was then used to calculate the TRAP which was expressed as nmoles antioxidant per mg LDL according to the equation below, in which it is assumed that trolox was capable of scavenging two peroxyl radicals per molecule in this system [18]:

$$\text{nmol antioxidant/mg LDL} = \frac{2(T_{\text{LDL}})(\text{nmol trolox added})}{(T_{\text{Trolox}})(\text{mg LDL added})}.$$

In some experiments the assay was conducted in the presence of linoleic acid supplemented with 200 μM 13-HPODE.

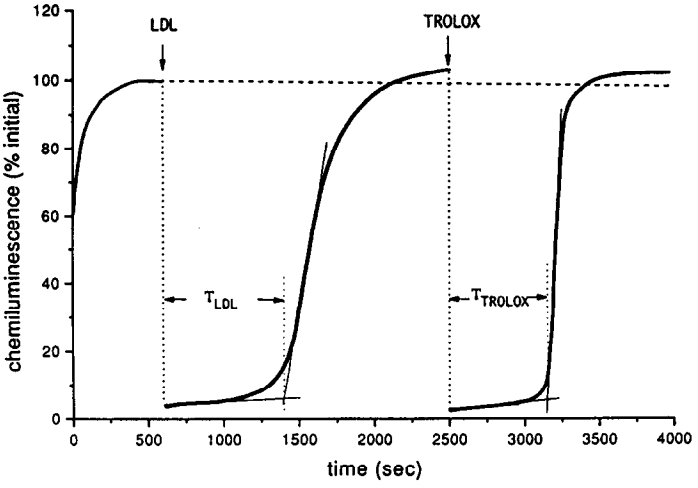

Fig. 1. Measurement of the inhibition of the peroxyl radical dependent oxidation of luminol by LDL. A sample of LDL (90 μL of 1 mg/mL) was added to a solution of luminol (0.87 mM) and linoleic acid (7 mM) in which oxidation had been previously initiated by the addition of ABAP. The chemiluminescence signal is expressed as a percentage of the signal at the beginning of the experiment and was typically in the range 20–30 mV. The length of time over which luminol oxidation was suppressed (T_{LDL}) was measured as shown. After the signal was restored a sample of trolox (1 nmol) was added and T_{trolox} measured.

Reagents. Luminol (5-amino-2,3-dihydro-1,4-phthalazinedione), DTPA (±)-α-tocopherol and sodium linoleate were purchased from the Sigma Chemical Co. (Poole, U.K.), ABAP from Polysciences (Warrington, PA, U.S.A.), Trolox from Aldrich (Gillingham, U.K.) and 13-HPODE from Cascade Biochem Ltd (Reading, U.K.).

RESULTS AND DISCUSSION

The effect of LDL and α-tocopherol on the ABAP dependent oxidation of luminol

It has been shown that treatment of LDL with ABAP results in oxidation of the lipid and that antioxidants present in LDL are capable of inhibiting ABAP dependent lipid peroxidation [13, 21]. Lipid peroxidation reactions may be followed by adding luminol to the reaction mixture which is oxidized to a chemiluminescent product, the formation of which can be monitored using a luminometer [20]. ABAP decomposes at a constant rate to form water soluble peroxyl radicals which we found could react with the luminol to give rise to chemiluminescence, however, in the presence of linoleic acid the signal increased approximately 4-fold. We have assumed that the rate of ABAP decomposition is not affected by the presence of linoleic acid and we calculate that 78 ± 7% (mean ± SD of four separate determinations) of the signal detected in this system can be attributed to linoleic acid derived peroxyl radicals. Under these conditions the peroxidation of linoleic acid provides a test system for the assessment of the activity of antioxidants as described previously for the TRAP assay [18, 20].

The results of a typical experiment are shown in Fig. 1 which shows the generation of a stable light signal arising from the ABAP dependent peroxidation of linoleic acid in the presence of luminol. The signal was inhibited by the addition of a sample of LDL (Fig. 1). After a period of time the light signal returns to a level similar to that observed before the addition of LDL. A standard amount (1 nmol) of the peroxyl radical scavenger trolox was then added and was used as an internal standard for the calculation of the TRAP value for a given sample (see Materials and Methods). The magnitude of the TRAP value is dependent upon the amount of antioxidant added and this is shown in Fig. 2 for α-tocopherol and its water soluble analogue trolox. In this figure the lag phase for trolox (Fig. 2a) is shown plotted as a function of the nominal amount of peroxyl radical scavenger added and it is clear that in both cases inhibition of the luminol signal is a linear function of the added antioxidant. The TRAP value for 1 nmol α-tocopherol was found to be 2.25 ± 0.13 (mean ± SD, N = 7) which indicates that, as expected, α-tocopherol is able to scavenge approximately the same number of peroxyl radicals as trolox [18]. The amount of LDL added, typically 0.07–0.2 nmol, contains, on average, 91–260 nmol of polyunsaturated fatty acid compared to 4025 nmol linoleic acid present in the incubation [12]. It is unlikely therefore, that donor dependent variation in the fatty acid composition of LDL would influence the efficiency of peroxyl radical scavengers in this assay, although we have not tested this assumption directly.

The azo initiator, ABAP, decomposes to form peroxyl radicals which are capable of propagating lipid peroxidation without a requirement for the decomposition of lipid hydroperoxides. It is unlikely, therefore, that lipid hydroperoxides present either

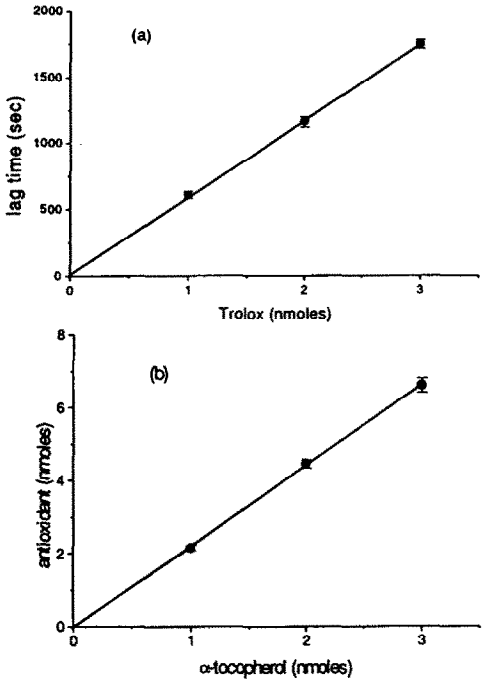

Fig. 2. Inhibition of the peroxyl radical dependent oxidation of luminol by α-tocopherol and trolox. The oxidation of luminol by ABAP in the presence of linoleic acid was established as described for Fig. 1. Samples of trolox and α-tocopherol were added in the quantities shown and the inhibition of the chemiluminescence signal measured. The lag phase for trolox is shown plotted in seconds and the antioxidant capacity (nmol trolox equivalents) for α-tocopherol was calculated as described in the Materials and Methods and is plotted as a function of the amount of α-tocopherol added. Each data point represents the mean ± SD for several independent experiments.

Table 1. The effect of 13-HPODE on the ABAP dependent oxidation of linoleic acid

Sample	Control Lag phase (sec)	+13-HPODE
α-Tocopherol	843 ± 21 (8)	831 ± 37 (7)
Trolox	748 ± 46 (11)	746 ± 58 (11)
LDL	370 ± 24 (12)	361 ± 19 (12)

The effect of 13-HPODE (200 μM) on the inhibition of the ABAP dependent oxidation of luminol by α-tocopherol, trolox and LDL (32 μg) was determined by supplementing the linoleic acid with the lipid hydroperoxide. The control sample contained linoleic acid which was supplied as essentially peroxide free. To aid comparison between samples the data are expressed as the length of the lag phase in seconds for 1 nmol antioxidant, in the case of trolox and α-tocopherol, and for 32 μg LDL and are reported as the mean ± SD. The number of determinations are shown in parenthesis.

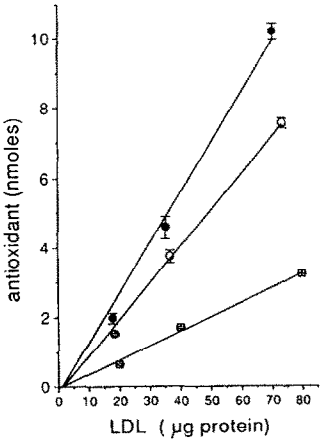

Fig. 3. The effect of LDL supplemented with different amounts of α-tocopherol on the peroxyl radical dependent oxidation of luminol. The conditions used were identical to those described for Fig. 1 except that LDL from a single donor was supplemented with α-tocopherol as described in the Materials and Methods. Different amounts of the supplemented LDL were added and the inhibition of luminol oxidation then determined. The data for three LDL preparations with an α-tocopherol content of 11.6 ± 1.43 nmol/mg (□), 35.32 ± 2.5 nmol/mg (○), and 53.5 ± 0.3 nmol/mg (●) is shown plotted as a function of the amount of LDL added to each experiment. Each data point represents the mean ± SD of at least three independent determinations.

in the linoleic acid or LDL influence the efficacy of antioxidants in this system. To test this assumption the linoleic acid used as a substrate for the peroxidation reaction was supplemented with 200 μM 13-HPODE and the TRAP value for α-tocopherol, trolox and a sample of LDL measured and compared with that obtained using the unsupplemented and essentially peroxide free linoleic acid. The results of this experiment are shown in Table 1 and demonstrate that lipid hydroperoxides do not affect the TRAP value for peroxyl radical scavengers or LDL. In marked contrast LDL supplemented with lipid hydroperoxides and treated with copper shows a greatly accelerated rate of oxidation [16].

In the next series of experiments the inhibition of the peroxyl radical dependent oxidation of luminol was measured as a function of the concentration of LDL isolated from plasma which had been supplemented, *ex vivo*, with different amounts of α-tocopherol (Fig. 3). It is clear that, as shown in Fig. 2 for the peroxyl radical scavengers α-tocopherol and trolox, the antioxidant activity of LDL is a linear function of the amount of LDL added suggesting that only the presence of peroxyl radical scavengers in LDL is detected in this assay. Supplementation of aliquots of the plasma isolated from this individual with α-tocopherol resulted in a progressive increase in the content of this antioxidant in the LDL purified from these plasma samples and this was associated with an increased resistance of the supplemented LDL sample to oxidation (Fig. 3). It has been

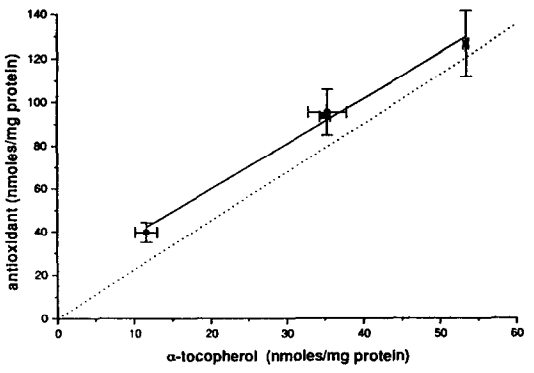

Fig. 4. The peroxyl radical scavenging activity of α-tocopherol in LDL. Four preparations of LDL isolated from the plasma from the same individual after supplementation with different amounts of α-tocopherol. The antioxidant content (nmol/mg) determined by the TRAP assay is shown plotted as a function of the different content of α-tocopherol in the LDL preparations. The data are represented as the mean ± SD for both the α-tocopherol determination and measurement of antioxidant content. The dotted line represents the data for authentic α-tocopherol extrapolated from Fig. 2.

Table 2. Oxidizability and antioxidant capacity of LDL isolated from different donors

LDL sample	Cu^{2+}oxidizability (min)	Vitamin E (nmol/mg)	Antioxidant capacity (nmol/mg LDL)
A	233 ± 37	11.7	25 ± 1.5
B	217 ± 35	9.2	28 ± 2.0
C	151 ± 9	11.9	34 ± 1.4
D	114 ± 20	11.4	32 ± 2.3
E	99 ± 2	12.3	32 ± 0.5
F	90 ± 4	13.9	28 ± 2
G	76 ± 0.6	10.5	31 ± 1
H	69 ± 12	10.0	27 ± 0.2
Mean	131 ± 63	11.4 ± 1.5	29.6 ± 3

LDL was isolated from eight individual donors and the α-tocopherol content measured by HPLC. The results are expressed as the mean of duplicate determinations from each sample. The oxidizability of each sample was determined after the addition of copper by monitoring the formation of conjugated dienes spectrophotometrically. The time taken to achieve half the maximum absorbance was used as an index of the oxidizability of each sample.

The results are expressed as the mean ± SD for three independent determinations. The antioxidant content of each LDL sample was measured as described in the text and is expressed as trolox equivalents. The final row shows the mean ± SD of the data for preparations A–H.

suggested that the efficacy of peroxyl radical scavengers in LDL may depend on their location within the LDL particle and it follows, therefore, that the efficacy of α-tocopherol added in an ethanolic solution directly to the assay mixture, as in Fig. 2, may differ from that of α-tocopherol incorporated within LDL, as in Fig. 3. To test for this possibility we have plotted the antioxidant capacity of the α-tocopherol supplemented LDL as a function of the α-tocopherol content of each sample and these results are shown in Fig. 4. Again antioxidant activity is a linear function of the nominal amount of α-tocopherol incorporated into LDL with a slope of 2.1 which is not significantly different to that found for α-tocopherol alone (Fig. 2). However it is important to note that the intercept of the line found with LDL does not pass through the origin suggesting that the peroxyl radical scavenging activity of LDL cannot be accounted for solely on the basis of α-tocopherol content. This could be due to the peroxyl radical scavenging activity of other small molecules such as the carotenes or ubiquinol or indeed the protein component of LDL [13, 17, 18].

The antioxidant capacity of LDL preparations isolated from individual donors and a comparison with the oxidizability of LDL in the presence of copper

The addition of copper to a solution of LDL which contains low levels of endogenous lipid hydroperoxides results in the oxidation of the lipid phase of the molecule and the formation of conjugated dienes [8, 10, 11]. These products of lipid peroxidation have a characteristic absorbance maximum at 234 nm and this property has been used to monitor the oxidation and determine the oxidizability of LDL samples isolated from individual donors [8, 10]. The rate of oxidation under these conditions probably depends upon both the lipid hydroperoxide and α-tocopherol content of the LDL molecule [15, 16]. In Table 2 we have compared the oxidizability of eight samples of LDL (from different individuals) measured in the presence of copper with the antioxidant capacity of the same LDL samples measured by the inhibition of the peroxyl radical dependent oxidation of luminol. The preparations are shown in order of increasing oxidizability, and, in agreement with several other laboratories, our data show that the initial levels of α-tocopherol do not correlate with the oxidizability of the LDL samples [8–11]. The α-tocopherol content of the eight individual LDL's represented here shows relatively little variation and the mean value of all the samples is equivalent to 5.35 ± 1 molecules of α-tocopherol per molecule LDL, in good agreement with that published in the literature [12]. The antioxidant capacity of LDL is also reported in Table 2 and, in contrast to the oxidizability measured in the presence of copper, shows relatively little variation amongst these individuals. The data presented in Fig. 4 suggests that the ability of α-tocopherol to scavenge peroxyl radicals is essentially identical whether added to the linoleic acid in the assay as an ethanolic solution or in LDL. The result also suggests that under these conditions the reaction can be considered as homogeneous with respect to α-tocopherol and peroxyl radicals.

It has already been shown that peroxyl radical scavengers thought to be in the "core" of the LDL particle, such as ubiquinol, are depleted rapidly by the same water soluble initiator, ABAP, used in this study. It is unlikely, therefore, that any potential peroxyl radical scavengers are not detected under

the conditions of the assay [13]. The TRAP assay is only able to detect peroxyl radical scavengers which are able to scavenge peroxyl radicals at a high rate and it could be argued, therefore, that we are underestimating the total capacity of the LDL particle to scavenge this oxidant. However, such scavengers are, by definition, inefficient and would be unlikely to make a significant contribution *in vivo* unless present in very high concentrations.

Assuming, as our data suggests, that the efficiency of the peroxyl radical scavenging of α-tocopherol is not dependent on how it is introduced into the assay we may calculate an average, theoretical, TRAP value for the α-tocopherol present in the LDL samples shown in Table 2. This procedure results in a mean TRAP value for α-tocopherol in these LDL samples of 25.6 which is approximately 86% of the average total TRAP value found for the LDL particle.

In summary, we have described a simple method for determination of the peroxyl radical scavenging capacity of human LDL samples which can be achieved with approximately 100 μg of LDL protein. The assay was constructed so that the contribution of the LDL lipid as a substrate for the peroxidation reaction was minimized thus allowing the total capacity of the LDL particle to scavenge peroxyl radicals to be measured. We suggest that the large variation between individuals in the rate of LDL oxidation promoted by copper is unlikely to be due to variations in the capacity of these samples to scavenge peroxyl radicals but could be due to other factors such as the endogenous lipid hydroperoxide content. This type of *ex vivo* assay can only measure factors intrinsic to the LDL particle and does not take into account the fact that *in vivo* the oxidizability and hence the potential atherogenicity of an LDL particle will also be affected by the synergistic interaction of water soluble antioxidants such as ascorbate with α-tocopherol [21, 24]. However, the strong epidemiological association between LDL cholesterol and increased risk for coronary heart disease suggest that the properties of isolated LDL are important in the development of coronary heart disease.

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